

## IMMUNOSTIMULATORY NUCLEIC ACIDS

### FIELD OF THE INVENTION

The present invention relates generally to immunostimulatory nucleic acids,  
5 compositions thereof and methods of using the immunostimulatory nucleic acids.

### RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119 to US Provisional Patent Application Nos. 60/156,113, filed September 25, 1999, 60/156,135, filed September 27, 10 1999, and 60/227,436, filed August 23, 2000, the entire contents of which are hereby incorporated by reference.

### BACKGROUND OF THE INVENTION

Bacterial DNA has immune stimulatory effects to activate B cells and natural killer cells, but vertebrate DNA does not (Tokunaga, T., et al., 1988. *Jpn. J. Cancer Res.* 79:682-686; Tokunaga, T., et al., 1984, *JNCI* 72:955-962; Messina, J.P., et al., 1991, *J. Immunol.* 147:1759-1764; and reviewed in Krieg, 1998, In: *Applied Oligonucleotide Technology*, C.A. Stein and A.M. Krieg, (Eds.), John Wiley and Sons, Inc., New York, NY, pp. 431-448). It is now understood that these immune stimulatory effects of bacterial DNA are a result of the presence of unmethylated CpG dinucleotides in particular base contexts (CpG motifs), which are common in bacterial DNA, but methylated and underrepresented in vertebrate DNA (Krieg et al, 1995 *Nature* 374:546-549; Krieg, 1999 *Biochim. Biophys. Acta* 93321:1-10). The immune stimulatory effects of bacterial DNA can be mimicked with synthetic oligodeoxynucleotides (ODN) containing these CpG motifs. Such CpG ODN have highly stimulatory effects on human and murine leukocytes, inducing B cell proliferation; cytokine and immunoglobulin secretion; natural killer (NK) cell lytic activity and IFN- $\gamma$  secretion; and activation of dendritic cells (DCs) and other antigen presenting cells to express costimulatory molecules and secrete cytokines, especially the Th1-like cytokines that are important in 20 promoting the development of Th1-like T cell responses. These immune stimulatory effects of native phosphodiester backbone CpG ODN are highly CpG specific in that the 25 effects are essentially abolished if the CpG motif is methylated, changed to a GpC, or 30 effects are essentially abolished if the CpG motif is methylated, changed to a GpC, or

otherwise eliminated or altered (Krieg et al, 1995 Nature 374:546-549; Hartmann et al, 1999 Proc. Natl. Acad. Sci USA 96:9305-10). Phosphodiester CpG ODN can be formulated in lipids, alum, or other types of vehicles with depot properties or improved cell uptake in order to enhance the immune stimulatory effects (Yamamoto et al, 1994 5 Microbiol. Immunol. 38:831-836; Gramzinski et al, 1998 Mol. Med. 4:109-118).

In early studies, it was thought that the immune stimulatory CpG motif followed the formula purine-purine-CpG-pyrimidine-pyrimidine (Krieg et al, 1995 Nature 374:546-549; Pisetsky, 1996 J. Immunol. 156:421-423; Hacker et al., 1998 EMBO J. 17:6230-6240; Lipford et al, 1998 Trends in Microbiol. 6:496-500). However, it is now 10 clear that mouse lymphocytes respond quite well to phosphodiester CpG motifs that do not follow this "formula" (Yi et al., 1998 J. Immunol. 160:5898-5906) and the same is true of human B cells and dendritic cells (Hartmann et al, 1999 Proc. Natl. Acad. Sci USA 96:9305-10; Liang, 1996 J. Clin. Invest. 98:1119-1129).

Several past investigators have looked at whether the nucleotide content of ODN 15 may have effects independently of the sequence of the ODN. Interestingly, antisense ODN have been found to be generally enriched in the content of GG, CCC, CC, CAC, and CG sequences, while having reduced frequency of TT or TCC nucleotide sequences compared to what would be expected if base usage were random (Smetsers et al., 1996 Antisense Nucleic Acid Drug Develop. 6:63-67). This raised the possibility that the 20 over-represented sequences may comprise preferred targeting elements for antisense oligonucleotides or visa versa. One reason to avoid the use of thymidine-rich ODN for antisense experiments is that degradation of the ODN by nucleases present in cells releases free thymidine which competes with <sup>3</sup>H-thymidine which is frequently used in experiments to assess cell proliferation (Matson et al., 1992 Antisense Research and 25 Development 2:325-330).

#### SUMMARY OF THE INVENTION

The present invention relates in part to pyrimidine rich (Py-rich) and in some 30 embodiments thymidine (T) rich immunostimulatory nucleic acids which do not require the presence of a CpG motif. The present invention also relates in part to the discovery that nucleic acids which contain a TG dinucleotide motif are also immunostimulatory. The invention is based in part on the unexpected finding that nucleic acid sequences

- which do not contain CpG motifs are immunostimulatory. It was discovered upon analysis of the immune stimulation properties of many nucleic acid sequences that these sequences may be Py-rich e.g., T-rich or that they may contain TG motifs. It was also discovered that these sequences preferentially activate non-rodent immune cells. The 5 Py-rich and TG sequences are only minimally immunostimulatory with respect to rodent immune cells, compared to non-rodent immune cells. Thus, it is possible according to the methods of the invention to induce an immune response in a non-rodent subject by administering Py-rich or TG immunostimulatory nucleic acids. The Py-rich and TG immunostimulatory nucleic acids of the invention may optionally include CpG motifs.
- 10 These findings have important implications for the clinical development of immunostimulatory CpG containing and non-CpG containing nucleic acids.

In one aspect the invention is a pharmaceutical composition comprising an effective amount for stimulating an immune response of isolated Py-rich or TG immunostimulatory nucleic acids, and a pharmaceutically acceptable carrier. In other 15 aspects the invention is a composition of matter, comprising an isolated Py-rich or TG immunostimulatory nucleic acid. In other embodiments, the immunostimulatory nucleic acid may be T-rich. In still other embodiments, the immunostimulatory nucleic acid may be T-rich and also have at least one TG motif.

Preferably the Py-rich nucleic acid is a T-rich nucleic acid. In some 20 embodiments the T-rich immunostimulatory nucleic acid is a poly T nucleic acid comprising 5' TTTT 3'. In yet other embodiments the poly T nucleic acid comprises 5' X<sub>1</sub> X<sub>2</sub>TTTX<sub>3</sub> X<sub>4</sub> 3' wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub> and X<sub>4</sub> are nucleotides. In some embodiments X<sub>1</sub>X<sub>2</sub> is TT and/or X<sub>3</sub>X<sub>4</sub> is TT. In other embodiments X<sub>1</sub>X<sub>2</sub> is selected from the group consisting of TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, CG, GT, GG, GA, and GC; 25 and/or X<sub>3</sub>X<sub>4</sub> is selected from the group consisting of TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, CG, GT, GG, GA, and GC.

The T-rich immunostimulatory nucleic acid may have only a single poly T motif or it may have a plurality of poly T nucleic acid motifs. In some embodiments the T-rich immunostimulatory nucleic acid comprises at least 2, at least 3, at least 4, at least 5, at 30 least 6, at least 7, or at least 8 T motifs. In other embodiments it comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 CpG motifs. In preferred embodiments the plurality of CpG motifs and poly T motifs are interspersed.

In yet other embodiments at least one of the plurality of poly T motifs comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, or at least 9 contiguous T nucleotide residues. In other embodiments the plurality of poly T motifs is at least 3 motifs and wherein at least 3 motifs each comprises at least 3 contiguous T nucleotide residues or the plurality of poly T motifs is at least 4 motifs and wherein the at least 4 motifs each comprises at least 3 contiguous T nucleotide residues.

In some cases the T-rich immunostimulatory nucleic acid may be free of poly T motifs but may rather comprise a nucleotide composition of greater than 25% T. In other embodiments the T-rich immunostimulatory nucleic acids have poly T motifs and also comprise a nucleotide composition of greater than 25% T. In preferred embodiments the T-rich immunostimulatory nucleic acid comprises a nucleotide composition of greater than 35% T, greater than 40% T, greater than 50% T, greater than 60% T, greater than 80% T, or greater than 90% T nucleotide residues. In important embodiments, the nucleic acid is at least 50% T.

The T-rich and TG immunostimulatory nucleic acids can have any length greater than 7 nucleotides, but in some embodiments can be between 8 and 100 nucleotide residues in length. In preferred embodiments the T-rich immunostimulatory nucleic acid comprises at least 20 nucleotides, at least 24 nucleotides, at least 27, nucleotides, or at least 30 nucleotides. In preferred embodiments, the TG immunostimulatory nucleic acid is between 15 and 25 nucleotides in length. The T-rich and TG immunostimulatory nucleic acids may be single stranded or double stranded.

In one preferred embodiment, the immunostimulatory nucleic acid has a T-rich region located in the middle of its length (i.e., an approximately equal number of nucleotides flank the T-rich region on the 5' and 3' ends).

The T rich nucleic acid in some embodiments is selected from the group consisting of SEQ ID NO: 59-63, 73-75, 142, 215, 226, 241, 267-269, 282, 301, 304, 330, 342, 358, 370-372, 393, 433, 471, 479, 486, 491, 497, 503, 556-558, 567, 694, 793-794, 797, 833, 852, 861, 867, 868, 882, 886, 905, 907, 908, and 910-913. In other embodiments the T rich nucleic acids are sequence selected from the group consisting of SEQ ID NO: 64, 98, 112, 146, 185, 204, 208, 214, 224, 233, 244, 246, 247, 258, 262, 263, 265, 270-273, 300, 305, 316, 317, 343, 344, 350, 352, 354, 374, 376, 392, 407, 411-

413, 429-432, 434, 435, 443, 474, 475, 498-501, 518, 687, 692, 693, 804, 862, 883, 884, 888, 890, and 891.

In other embodiments the Py-rich immunostimulatory nucleic acid is a C-rich nucleic acid. An immunostimulatory C-rich nucleic acid is a nucleic acid including at 5 least one and preferably at least 2 poly-C regions or which includes 50% or greater C nucleotides.

The Py-rich and TG immunostimulatory nucleic acids may include one or more CpG motifs. The motifs may be methylated or unmethylated. In other embodiments the Py-rich and TG immunostimulatory nucleic acids are free of one or more CpG 10 dinucleotides.

In other embodiments the Py-rich and TG immunostimulatory nucleic acids also include poly-A, poly G, and/or poly C motifs. In yet other embodiments the Py-rich or TG immunostimulatory nucleic acid is free of two poly C sequences of at least 3 contiguous C nucleotide residues or is free of two poly A sequences of at least 3 15 contiguous A nucleotide residues. In other embodiments the Py-rich or TG immunostimulatory nucleic acid comprises a nucleotide composition of greater than 25% C or greater than 25% A. In yet other embodiments the Py-rich or TG immunostimulatory nucleic acid is free of poly-C sequences, poly-G sequences or poly-A sequences.

A poly G nucleic acid in some embodiments is selected from the group consisting 20 of SEQ ID NO: 5, 6, 73, 215, 267-269, 276, 282, 288, 297-299, 355, 359, 386, 387, 444, 476, 531, 557-559, 733, 768, 795, 796, 914-925, 928-931, 933-936, and 938. In other embodiments the poly G nucleic acid includes a sequence selected from the group consisting of SEQ ID NO: 67, 80-82, 141, 147, 148, 173, 178, 183, 185, 214, 224, 264, 25 265, 315, 329, 434, 435, 475, 519, 521-524, 526, 527, 535, 554, 565, 609, 628, 660, 661, 662, 725, 767, 825, 856, 857, 876, 892, 909, 926, 927, 932, and 937.

According to another aspect of the invention, the immunostimulatory nucleic acids may be defined as those which possess a TG motif, herein referred to as TG 30 immunostimulatory nucleic acids. The TG nucleic acid in one embodiment contains at least one TG dinucleotide having a sequence including at least the following formula: 5'N<sub>1</sub>X<sub>1</sub>TGX<sub>2</sub>N<sub>2</sub>3'. In related embodiments, N<sub>1</sub> is a nucleic acid sequence composed of a number of nucleotides ranging from (11-N<sub>2</sub>) to (21-N<sub>2</sub>) and N<sub>2</sub> is a nucleic acid sequence

composed of a number of nucleotides ranging from (11-N<sub>1</sub>) to (21-N<sub>1</sub>). In a preferred embodiment, X<sub>2</sub> is thymidine.

In other embodiments, the TG nucleic acid has at least the following formula: 5' X<sub>1</sub> X<sub>2</sub>TGX<sub>3</sub> X<sub>4</sub> 3'. In yet another embodiment, the TG nucleic acid comprises the 5 following sequence: 5'N<sub>1</sub>X<sub>1</sub>X<sub>2</sub>TGX<sub>3</sub>X<sub>4</sub>N<sub>2</sub>3'. In a related embodiment, N<sub>1</sub> is a nucleic acid sequence composed of a number of nucleotides ranging from (9-N<sub>2</sub>) to (19-N<sub>2</sub>) and N<sub>2</sub> is a nucleic acid sequence composed of a number of nucleotides ranging from (9-N<sub>1</sub>) to (19-N<sub>1</sub>). In one preferred embodiment, X<sub>3</sub> is thymidine. X<sub>1</sub>X<sub>2</sub> are nucleotides which may be selected from the group consisting of GT, GG, GA, AA, AT, AG, CT, CA, CG, 10 TA and TT, and X<sub>3</sub>X<sub>4</sub> are nucleotides which may be selected from the group consisting of TT, CT, AT, AG, CG, TC, AC, CC, TA, AA, and CA. In some preferred embodiments, X<sub>3</sub> is a thymidine. In important embodiments, X<sub>3</sub>X<sub>4</sub> are nucleotides selected from the group consisting of TT, TC, TA and TG. In other embodiments X<sub>1</sub>X<sub>2</sub> are GA or GT and X<sub>3</sub>X<sub>4</sub> are TT. In yet other embodiments X<sub>1</sub> or X<sub>2</sub> or both are purines 15 and X<sub>3</sub> or X<sub>4</sub> or both are pyrimidines or X<sub>1</sub>X<sub>2</sub> are GpA and X<sub>3</sub> or X<sub>4</sub> or both are pyrimidines. In one embodiment X<sub>2</sub> is a T and X<sub>3</sub> is a pyrimidine.

In one embodiment the 5' X<sub>1</sub> X<sub>2</sub>TGX<sub>3</sub> X<sub>4</sub> 3' sequence of the TG nucleic acid or the entire length or some fragment thereof of the TG nucleic acid is a non-palindromic sequence, and in other embodiments it is a palindromic sequence.

20 In some preferred embodiments, the TG nucleic acid is also T-rich.

The Py-rich and TG immunostimulatory nucleic acids in some embodiments have a nucleotide backbone which includes at least one backbone modification, such as a phosphorothioate modification. The nucleotide backbone may be chimeric, or preferably the nucleotide backbone is entirely modified. In one preferred embodiment, the 25 immunostimulatory nucleic acid has a poly T motif and a phosphorothioate backbone.

In another aspect the invention is a composition of an immunostimulatory nucleic acid, in the form of a Py-rich or a TG nucleic acid, and an antigen, wherein the nucleic acid is free of unmethylated CpG motifs.

Another composition of the invention is a Py-rich or TG immunostimulatory 30 nucleic acid and an anti-microbial agent, wherein the Py-rich or TG nucleic acid is free of unmethylated CpG motifs. Preferably the anti-microbial agent is selected from the

group consisting of an anti-viral agent, an anti-parasitic agent, an anti-bacterial agent and an anti-fungal agent.

A composition of a sustained release device including a Py-rich and/or TG immunostimulatory nucleic acid, wherein the Py-rich and/or TG nucleic acid is free of unmethylated CpG motifs, is provided according to another aspect of the invention.

The invention also includes nutritional supplements of a Py-rich or TG immunostimulatory nucleic acid in a delivery device selected from the group consisting of a capsule, a pill, and a sublingual tablet, wherein the Py-rich or TG nucleic acid is free of unmethylated CpG motifs.

It should be understood that when it is useful to administer a Py-rich e.g., poly T, T-rich, C-rich, or TG oligonucleotide and a CpG oligonucleotide, it may also be desirable to co-administer a Py-rich or a TG oligonucleotide together with a physically separate CpG, Py-rich or TG oligonucleotide. Alternatively, the CpG, Py-rich or TG motif may be present on the same contiguous nucleic acid as the Py-rich or TG oligonucleotide. In yet a further embodiment, all or some combination of Py-rich, TG and CpG nucleic acids may be co-administered either on separate nucleic acids or in the same nucleic acid molecule. By co-administer it is intended that the nucleic acids be administered close enough in time to one another to achieve a combined benefit of both oligonucleotides, preferably more than the benefit achieved by administering each of the oligonucleotides alone at the same dose.

CpG oligonucleotides have, in general, the formula 5'X<sub>1</sub>X<sub>2</sub>CGX<sub>3</sub>X<sub>4</sub>3', wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub> and X<sub>4</sub> are nucleotides and wherein at least the C of CpG is unmethylated. Preferred CpG oligonucleotides are 8-100 nucleotides in length and have modified back bones. Particular structures are detailed in the published PCT applications, U.S. applications and references cited herein, the disclosures of which are incorporated herein in their entirety. In one embodiment, the CpG oligonucleotide is free of poly T and TG motifs and is not T-rich.

In other embodiments, the CpG oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 1, 3, 4, 14-16, 18-24, 28, 29, 33-46, 49, 50, 52-56, 58, 64-67, 69, 71, 72, 76-87, 90, 91, 93, 94, 96, 98, 102-124, 126-128, 131-133, 136-141, 146-150, 152-153, 155-171, 173-178, 180-186, 188-198, 201, 203-214, 216-220, 223, 224, 227-240, 242-256, 258, 260-265, 270-273, 275, 277-281, 286-287, 292, 295-296,

300, 302, 305-307, 309-312, 314-317, 320-327, 329, 335, 337-341, 343-352, 354, 357,  
361-365, 367-369, 373-376, 378-385, 388-392, 394, 395, 399, 401-404, 406-426, 429-  
433, 434-437, 439, 441-443, 445, 447, 448, 450, 453-456, 460-464, 466-469, 472-475,  
477, 478, 480, 483-485, 488, 489, 492, 493, 495-502, 504-505, 507-509, 511, 513-529,  
5 532-541, 543-555, 564-566, 568-576, 578, 580, 599, 601-605, 607-611, 613-615, 617,  
619-622, 625-646, 648-650, 653-664, 666-697, 699-706, 708, 709, 711-716, 718-732,  
736, 737, 739-744, 746, 747, 749-761, 763, 766-767, 769, 772-779, 781-783, 785-786,  
7900792, 798-799, 804-808, 810, 815, 817, 818, 820-832, 835-846, 849-850, 855-859,  
862, 865, 872, 874-877, 879-881, 883-885, 888-904, and 909-913.

10 In another embodiment, the Py-rich or TG oligonucleotide is free of a CpG motifs. This embodiment of the invention also involves pharmaceutical compositions and kits which contain both a CpG oligonucleotide (which can be free of poly T and TG motifs and not be T-rich) and a Py-rich and/or TG oligonucleotide physically separate from the CpG oligonucleotide. The pharmaceutical preparations are in effective amounts  
15 and typically include pharmaceutically acceptable carriers, all as set forth in detail herein with respect to Py-rich and TG oligonucleotides. The kits include at least one container containing an oligonucleotide which is a Py-rich or TG oligonucleotide (or some combination thereof). The same container, or in other embodiments, a second container, may contain an oligonucleotide with a CpG motif, which may be free of Py-rich and/or  
20 TG motifs. The kit also contains instructions for administering the oligonucleotides to a subject. The kits also may include a container containing a solvent or a diluent.

In summary, as if fully recited herein, a CpG oligonucleotide physically separate from the Py-rich or TG oligonucleotide can be used together with the Py-rich or TG oligonucleotides in the methods, compositions and products described above.

25 The invention relates in other aspects to immunostimulatory oligonucleotides which have chimeric backbones and which do not require the presence of a CpG motif. The invention is based in part on the discovery that nucleic acid sequences which did not contain CpG motifs were immunostimulatory, and that those which have chimeric backbones have unexpectedly enhanced immune stimulating properties. Thus the  
30 invention in one aspect relates to a composition of an oligonucleotide having a formula:  
5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3', wherein Y<sub>1</sub> and Y<sub>2</sub> are, independent of one another, nucleic acid molecules having between 1 and 10 nucleotides, wherein Y<sub>1</sub> includes at least one

modified internucleotide linkage and Y<sub>2</sub> includes at least one modified internucleotide linkage and wherein N<sub>1</sub> and N<sub>2</sub> are nucleic acid molecules, each independent of one another, having between 0 and 5 nucleotides, but wherein N<sub>1</sub>ZN<sub>2</sub> has at least 6 nucleotides in total and wherein the nucleotides of N<sub>1</sub>ZN<sub>2</sub> have a phosphodiester backbone, and wherein Z is an immunostimulatory nucleic acid motif but does not include a CG. In one embodiment Z is a nucleic acid sequence selected from the group consisting of TTTT, TG, and a sequence wherein at least 50% of the bases of the sequence are Ts.

In some embodiments Y<sub>1</sub> and/or Y<sub>2</sub> have between 3 and 8 nucleotides. In other 10 embodiments Y<sub>1</sub> and/or Y<sub>2</sub> are comprised of at least three Gs, at least four Gs, least seven Gs, or all Gs. In other embodiments Y<sub>1</sub> and/or Y<sub>2</sub> are selected from the group consisting of TCGTCG, TCGTCGT, and TCGTCGTT (SEQ ID NO:1145). In yet other embodiments Y<sub>1</sub> and/or Y<sub>2</sub> include at least one, two, three, four, or five poly-A, poly-T, or poly-C sequences.

15 The center nucleotides (N<sub>1</sub>ZN<sub>2</sub>) of the formula Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> have phosphodiester internucleotide linkages and Y<sub>1</sub> and Y<sub>2</sub> have at least one modified internucleotide linkage. In some embodiments Y<sub>1</sub> and/or Y<sub>2</sub> have at least two modified internucleotide linkages. In other embodiments Y<sub>1</sub> and/or Y<sub>2</sub> have between two and five modified 20 internucleotide linkages. In yet other embodiments Y<sub>1</sub> has two modified internucleotide linkages and Y<sub>2</sub> has five modified internucleotide linkages or Y<sub>1</sub> has five modified internucleotide linkages and Y<sub>2</sub> has two modified internucleotide linkages. The modified internucleotide linkage, in some embodiments is a phosphorothioate modified linkage, a phosphorodithioate modified linkage or a p-ethoxy modified linkage.

25 Portions of the formula Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> may optionally form a palindrome. Thus, in some embodiments the nucleotides of N<sub>1</sub>ZN<sub>2</sub> form a palindrome. In some embodiments the palindrome is not a direct repeat. In yet other embodiments the nucleotides of N<sub>1</sub>ZN<sub>2</sub> do not form a palindrome.

According to other embodiments N<sub>1</sub>ZN<sub>2</sub> has a sequence of nucleotides selected 30 from the group consisting of GATTTTATCGTC (SEQ ID NO: 1098) ; TCGATTTTCGA (SEQ ID NO: 1099); TCATTTTATGTA (SEQ ID NO: 1100); GTTTTTACGAC (SEQ ID NO: 1101); TCAATTITITGA (SEQ ID NO: 1102); ACGTTTTACGT (SEQ ID NO: 1103); TCGTTTTACGA (SEQ ID NO: 1104);

TCGATTTTACGTCGA (SEQ ID NO: 1105); AATTCTTTAACGTT (SEQ ID NO: 1106); TCGTTTTAACGA (SEQ ID NO: 1107); ACGTTTTAACGT (SEQ ID NO: 1108); GATTTTATCGTC (SEQ ID NO: 1109); GACGATTTTCGTC (SEQ ID NO: 1110); GATTTAGCTCGTC (SEQ ID NO: 1111); GATTTTACGTC (SEQ ID NO: 1112); ATTTATCGT (SEQ ID NO: 1113); AACGATTTTCGTT (SEQ ID NO: 1114); TCACTTTGTA (SEQ ID NO: 1115); TCGTATTAA (SEQ ID NO: 1116); ACTTTTGTACCGGT (SEQ ID NO: 1117); TCGATTTTCGACGTCGA (SEQ ID NO: 1118); ACGATTTTCGT (SEQ ID NO: 1119); GATGATCGTC (SEQ ID NO: 1120); TCGATGTCGA (SEQ ID NO: 1121); TCATGTATGA (SEQ ID NO: 1122);  
10 GTGTTACGAC (SEQ ID NO: 1123); TCAATGTTGA (SEQ ID NO: 1124);  
ACGTGTACGT (SEQ ID NO: 1125); TCGTGTACGA (SEQ ID NO: 1126);  
TCGATGTACGTCGA (SEQ ID NO: 1127); AATGTTAACGTT (SEQ ID NO: 1128);  
TCGTGTTAACGA (SEQ ID NO: 1129); ACGTGTAAACGT (SEQ ID NO: 1130);  
GATGTATCGTC (SEQ ID NO: 1131); GACGATGTCGTC (SEQ ID NO: 1132);  
15 GATGAGCTCGTC (SEQ ID NO: 1133); GATGTACGTC (SEQ ID NO: 1134);  
ATGATCGT (SEQ ID NO: 1135); AACGATGTCGTT (SEQ ID NO: 1136);  
TCACTGGTGA (SEQ ID NO: 1137); TCGTATGA (SEQ ID NO: 1138);  
ACTGGTACCGGT (SEQ ID NO: 1139); TCGATGTCGACGTCGA (SEQ ID NO:  
1140); and ACGATGTCGT (SEQ ID NO: 1141).

20 The composition may optionally include a pharmaceutical carrier and/or be formulated in a delivery device. In some embodiments the delivery device is selected from the group consisting of cationic lipids, cell permeating proteins, and sustained release devices. In one preferred embodiment the sustained release device is a biodegradable polymer. In another embodiment the sustained release device is a  
25 microparticle.

In another aspect the invention is a composition of an immunostimulatory oligonucleotide having the formula  $Y_1N_1ZN_2Y_2$ , and an antigen.

Another composition of the invention is an immunostimulatory oligonucleotide having the formula  $Y_1N_1ZN_2Y_2$ , and an anti-microbial therapeutic agent. Preferably the  
30 anti-microbial therapeutic agent is selected from the group consisting of an anti-viral agent, an anti-parasitic agent, an anti-bacterial agent, or an anti-fungal agent.

A composition of a sustained release device including an immunostimulatory oligonucleotide having the formula Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub>, is provided according to another aspect of the invention.

The invention also includes nutritional supplements of an immunostimulatory 5 oligonucleotide having the formula Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub>, in a delivery device selected from the group consisting of a capsule, a sublingual tablet, and a pill.

In another aspect the compositions described above also include an 10 immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence wherein the immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence has a 15 different sequence than the oligonucleotide comprising 5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3'.

In some embodiments the immunostimulatory nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence has a completely phosphodiester backbone and in other embodiments the immunostimulatory 15 nucleic acid having an unmethylated CG dinucleotide, a TG dinucleotide or a Py-rich sequence has a modified backbone, which optionally may have internucleotide linkages selected from the group consisting of phosphorothioate, phosphorodithioate, and p-ethoxy.

In one embodiment immunostimulatory nucleic acid having an unmethylated CG 20 dinucleotide has a formula comprising: 5' X<sub>1</sub>X<sub>2</sub>CGX<sub>3</sub>X<sub>4</sub> 3' wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub> and X<sub>4</sub> are nucleotides. In other embodiments the immunostimulatory nucleic acid sequence includes at least the following formula: 5' TCNTX<sub>1</sub>X<sub>2</sub>CGX<sub>3</sub>X<sub>4</sub> 3' wherein N is a nucleic acid sequence composed of from about 0-25 nucleotides, wherein at least one nucleotide has a modified internucleotide linkage, and whercin the nucleic acid has less than or 25 equal to 100 nucleotides. According to some embodiments X<sub>1</sub>X<sub>2</sub> are nucleotides selected from the group consisting of: GT, GG, GA and AA and X<sub>3</sub>X<sub>4</sub> are nucleotides selected from the group consisting of: TT, CT or GT. In a preferred embodiment X<sub>1</sub>X<sub>2</sub> are GA and X<sub>3</sub>X<sub>4</sub> are TT.

In another embodiment the immunostimulatory nucleic acid sequence having an unmethylated CG dinucleotide includes at least one of the following sequences: 30 ATCGACTCTCGAGCGTTCTC (SEQ ID No.15); TCCATGTCGGTCCCTGCTGAT (SEQ ID No. 32); TCCATGTCGGTZCTGATGCT (SEQ ID No. 31);

ATCGACTCTCGAGCGTTZTC (SEQ ID No. 18); TCCATGTCGGTCCTGATGCT (SEQ ID No. 28); GGGGGG (SEQ ID No. 12); TCCATGACGGTCCTGATGCT (SEQ ID No. 35); TCCATGGCGGTCTGATGCT (SEQ ID No. 34);  
5 TCCATGACGTTCTGATGCT (SEQ ID No. 7); TCCATGTCGTTCTGATGCT (SEQ ID No. 38); GGGGTCAGTCTTGACGGGG (SEQ ID No. 41);  
TCCATGTCGCTCCTGATGCT (SEQ ID No. 37); TCCATGTCGATCCTGATGCT (SEQ ID No. 36); TCCATGCCGGTCCTGATGCT (SEQ ID No. 33);  
TCCATAACGTTCTGATGCT (SEQ ID No. 3); TCCATGACGTTCTGATGCT (SEQ ID No. 7); TCCATGACGTCCTGATGCT (SEQ ID No. 39);  
10 TCCATCACGTGCCTGATGCT (SEQ ID No. 48); TCCATGACGTTCTGACGTT (SEQ ID No. 10); ATGACGTTCTGACGTT (SEQ ID No. 70);  
TCTCCCAGCGCGCGCCAT (SEQ ID No. 72); TCCATGTCGTTCTGTCGTT (SEQ ID No. 73); TCCATAGCGTTCTAGCGTT (SEQ ID No. 74);  
15 TCCTGACGTTCTGACGTT (SEQ ID No. 76); TCCTGTCGTTCTGTCGTT (SEQ ID No. 77); TCCTGTCGTTCTGTCGTT (SEQ ID No. 52);  
TCCTTGTGCGTCTCTGTCGTT (SEQ ID No. 121); TCCTGTCGTTTTTGTCGTT (SEQ ID No. 208); TCGTCGCTGTTGTCGTTCTT (SEQ ID No. 120);  
TCCATGCGTTGCGTTGCGTT (SEQ ID No. 81); TCCACGACGTTTCGACGTT (SEQ ID No. 82); TCGTCGTTGTCGTTGTCGTT (SEQ ID No. 47);  
20 TCGTCGTTGTCGTTGTCGTT (SEQ ID No. 46);  
TCGTCGTTGTCGTTGTCGTT (SEQ ID No. 49);  
GCGTGCCTGTCGTTGTCGTT (SEQ ID No. 56); TGTCGTTGTCGTTGTCGTT (SEQ ID No. 48); TGTCGTTGTCGTTGTCGTT (SEQ ID No. 84);  
TGTCGTTGTCGTTGTCGTT (SEQ ID No. 50); TCGTCGTCGTCGTT (SEQ ID No. 51); and TGTCGTTGTCGTT (SEQ ID No. 85). In another embodiment the  
25 immunostimulatory nucleic acid having a Py-rich or TG sequence is a nucleic acid as described above.

In another aspect the invention relates to pharmaceutical compositions and kits which contain both an oligonucleotide having the formula  $Y_1N_1ZN_2Y_2$  and a CpG 30 oligonucleotide (which optionally may be free of poly T and TG motifs and not be Py-rich), a Py-rich and/or TG oligonucleotide physically separate from the oligonucleotide having the formula  $Y_1N_1ZN_2Y_2$ . The pharmaceutical preparations are in effective

amounts and typically include pharmaceutically acceptable carriers, all as set forth in detail herein. The kits include at least one container containing an oligonucleotide having the formula  $Y_1N_1ZN_2Y_2$ . The same container, or in other embodiments, a second container, may contain an oligonucleotide with a CpG motif, which optionally may be free of Py-rich and/or TG motifs and/or a Py-rich or TG oligonucleotide (or some combination thereof). The kit also contains instructions for administering the oligonucleotides to a subject. The kits also may include a container containing a solvent or a diluent.

In summary, as if fully recited herein, an oligonucleotide having the formula  $Y_1N_1ZN_2Y_2$  which is physically separate from the CpG, Py-rich or TG oligonucleotide can be used together with the CpG, Py-rich, TG oligonucleotides, in the methods, compositions and products described herein.

In another aspect the invention relates to a pharmaceutical composition including at least two oligonucleotides of the invention, wherein the at least two oligonucleotides have different sequences from one another and a pharmaceutically acceptable carrier.

A vaccine formulation is provided according to another aspect of the invention. The vaccine includes any of the compositions of the invention in combination with an antigen.

According to another aspect of the invention a method of stimulating an immune response is provided. The method involves administering a Py-rich or a TG immunostimulatory nucleic acid to a non-rodent subject in an amount effective to induce an immune response in the non-rodent subject. Preferably the Py-rich or TG immunostimulatory nucleic acid is administered orally, locally, in a sustained release device, mucosally to a mucosal surface, systemically, parenterally, or intramuscularly.

When the Py-rich or TG immunostimulatory nucleic acid is administered to the mucosal surface it may be delivered in an amount effective for inducing a mucosal immune response or a systemic immune response. In preferred embodiments the mucosal surface is selected from the group consisting of an oral, nasal, rectal, vaginal, and ocular surface.

In some embodiments the method includes exposing the subject to an antigen wherein the immune response is an antigen-specific immune response. The antigen may be encoded by a nucleic acid vector which can be delivered to the subject. In some

embodiments the antigen is selected from the group consisting of a tumor antigen, a viral antigen, a bacterial antigen, a parasitic antigen and a peptide antigen.

Py-rich and TG immunostimulatory nucleic acids are capable of provoking a broad spectrum of immune response. For instance these immunostimulatory nucleic acids can be used to redirect a Th2 to a Th1 immune response. Py-rich and TG nucleic acids may also be used to activate an immune cell, such as a leukocyte, a dendritic cell, and an NK cell. The activation can be performed *in vivo*, *in vitro*, or *ex vivo*, i.e., by isolating an immune cell from the subject, contacting the immune cell with an effective amount to activate the immune cell of the Py-rich or TG immunostimulatory nucleic acid and re-administering the activated immune cell to the subject. In some embodiments the dendritic cell expresses a cancer antigen. The dendritic cell can be exposed to the cancer antigen *ex vivo*.

The immune response produced by Py-rich or TG nucleic acids may also result in induction of cytokine production, e.g., production of IL-6, IL-12, IL-18 TNF, IFN- $\alpha$  and IFN- $\gamma$ .

In still another embodiment, the Py-rich and TG nucleic acids are useful for treating cancer. The Py-rich and TG nucleic acids are also useful according to other aspects of the invention in preventing cancer (e.g., reducing a risk of developing cancer) in a subject at risk of developing a cancer. The cancer may be selected from the group consisting of biliary tract cancer, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, gastric cancer, intraepithelial neoplasms, lymphomas, liver cancer, lung cancer (e.g. small cell and non-small cell), melanoma, neuroblastomas, oral cancer, ovarian cancer, pancreas cancer, prostate cancer, rectal cancer, sarcomas, thyroid cancer, and renal cancer, as well as other carcinomas and sarcomas. In some important embodiments, the cancer is selected from the group consisting of bone cancer, brain and CNS cancer, connective tissue cancer, esophageal cancer, eye cancer, Hodgkin's lymphoma, larynx cancer, oral cavity cancer, skin cancer, and testicular cancer.

Py-rich and TG nucleic acids may also be used for increasing the responsiveness of a cancer cell to a cancer therapy (e.g., an anti-cancer therapy), optionally when the Py-rich or TG immunostimulatory nucleic acid is administered in conjunction with an anti-cancer therapy. The anti-cancer therapy may be a chemotherapy, a vaccine (e.g., an *in vitro* primed dendritic cell vaccine or a cancer antigen vaccine) or an antibody based

therapy. This latter therapy may also involve administering an antibody specific for a cell surface antigen of, for example, a cancer cell, wherein the immune response results in antigen dependent cellular cytotoxicity (ADCC). In one embodiment, the antibody may be selected from the group consisting Ributaxin, Herceptin, Quadramet, Panorex, 5 IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior t6, MDX-210, MDX-11, MDX-22, OV103, 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 10 676, Monopharm-C, 4B5, ior egf.r3, ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART 1D10 Ab, SMART ABL 364 Ab and ImmuRAIT-CEA.

Thus, according to some aspects of the invention, a subject having cancer or at risk of having a cancer is administered an immunostimulatory nucleic acid and an anti-cancer therapy. In some embodiments, the anti-cancer therapy is selected from the group consisting of a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine. The chemotherapeutic agent may be selected from the group consisting of methotrexate, vincristine, adriamycin, cisplatin, non-sugar containing chloroethylnitrosoureas, 5-fluorouracil, mitomycin C, bleomycin, doxorubicin, dacarbazine, taxol, fragyline, Meglamine GLA, valrubicin, carmustaine and poliferposan, MMI270, BAY 12-9566, RAS famesyl transferase inhibitor, famesyl 15 transferase inhibitor, MMP, MTA/LY231514, LY264618/Lometexol, Glamolec, CI-994, TNP-470, Hycamtin/Topotecan, PKC412, Valspodar/PSC833, 20 Novantrone/Mitroxantrone, Metaret/Suramin, Batimastat, E7070, BCH-4556, CS-682, 9- AC, AG3340, AG3433, Incel/VX-710, VX-853, ZD0101, ISI641, ODN 698, TA 25 2516/Marmistat, BB2516/Marmistat, CDP 845, D2163, PD183805, DX8951f, Lemonal DP 2202, FK 317, Picibanil/OK-432, AD 32/Valrubicin, Metastron/strontium derivative, Temodal/Temozolomide, Evacet/liposomal doxorubicin, Yewtaxan/Placlitaxel, 30 Taxol/Paclitaxel, Xelod/Capecitabine, Furtulon/Doxifluridine, Cyclopax/oral paclitaxel, Oral Taxoid, SPU-077/Cisplatin, HMR 1275/Flavopiridol, CP-358 (774)/EGFR, CP-609 (754)/RAS oncogene inhibitor, BMS-182751/oral platinum, UFT(Tegafur/Uracil), Ergamisol/Levamisole, Eniluracil/776C85/5FU enhancer, Campto/Levamisole, 35 Camptosar/Irinotecan, Turnodex/Raltrexed, Leustatin/Cladribine, Paxex/Paclitaxel, Doxil/liposomal doxorubicin, Caelyx/liposomal doxorubicin, Fludara/Fludarabine,

- Pharmarubicin/Epirubicin, DepoCyt, ZD1839, LU 79553/Bis-Napthalimide, LU 103793/Dolastain, Caetyx/liposomal doxorubicin, Gemzar/Gemcitabine, ZD 0473/Anormed, YM 116, Iodine seeds, CDK4 and CDK2 inhibitors, PARP inhibitors, D4809/Dexifosamide, Ifes/Mesnex/Ifosamide, Vumon/Teniposide,
- 5 Paraplatin/Carboplatin, Plantinol/cisplatin, Vepeside/Etoposide, ZD 9331, Taxotere/Docetaxel, prodrug of guanine arabinoside, Taxane Analog, nitrosoureas, alkylating agents such as melphelan and cyclophosphamide, Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorambucil, Cytarabine HCl, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Flouxuridine,
- 10 Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o,p'-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine
- 15 (m-AMSA), Azacitidine, Erythropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate, but it is not so limited.

- The immunotherapeutic agent may be selected from the group consisting of
- 20 Ributaxin, Herceptin, Quadramet, Panorex, IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior t6, MDX-210, MDX-11, MDX-22, OV103, 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 676, Monopharm-C, 4B5, ior
- 25 egf.r3, ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART 1D10 Ab, SMART ABL 364 Ab and ImmuRAIT-CEA, but it is not so limited.

- The cancer vaccine may be selected from the group consisting of EGF, Anti-idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melaccine, peptide antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BCG vaccine, TA-HPV, TA-CIN, DISC-virus and ImmuCyst/TheraCys, but it is not so limited.

In still another embodiment of the methods directed to preventing or treating cancer, the subject may be further administered interferon- $\alpha$ .

- The invention in other aspects relates to methods for preventing disease in a subject. The method involves administering to the subject a Py-rich or a TG immunostimulatory nucleic acid on a regular basis to promote immune system responsiveness to prevent disease in the subject. Examples of diseases or conditions sought to be prevented using the prophylactic methods of the invention include microbial infections (e.g., sexually transmitted diseases) and anaphylactic shock from food allergies.
- 10 In other aspects, the invention is a method for inducing an innate immune response by administering to the subject a Py-rich or a TG immunostimulatory nucleic acid in an amount effective for activating an innate immune response.
- According to another aspect of the invention a method for treating or preventing a viral or retroviral infection is provided. The method involves administering to a subject having or at risk of having a viral or retroviral infection, an effective amount for treating or preventing the viral or retroviral infection of any of the compositions of the invention. In some embodiments the virus is caused by a hepatitis virus, HIV, hepatitis B, hepatitis C, herpes virus, or papillomavirus.
- 20 A method for treating or preventing a bacterial infection is provided according to another aspect of the invention. The method involves administering to a subject having or at risk of having a bacterial infection, an effective amount for treating or preventing the bacterial infection of any of the compositions of the invention. In one embodiment the bacterial infection is due to an intracellular bacteria.
- 25 In another aspect the invention is a method for treating or preventing a parasite infection by administering to a subject having or at risk of having a parasite infection, an effective amount for treating or preventing the parasite infection of any of the compositions of the invention. In one embodiment the parasite infection is due to an intracellular parasite. In another embodiment the parasite infection is due to a non-helminthic parasite.
- 30 In some embodiments the subject is a human and in other embodiments the subject is a non-human vertebrate selected from the group consisting of a dog, cat, horse, cow, pig, goat, fish, monkey, chicken, and sheep.

In yet another aspect, the invention is a method for treating or preventing asthma, by administering to a subject having or at risk of having asthma, an effective amount for treating or preventing the asthma of any of the compositions of the invention. In one embodiment the asthma is allergic asthma.

5       In another aspect the invention relates to a method for treating or preventing allergy. The method involves administering to a subject having or at risk of having allergy, an effective amount for treating or preventing the allergy of any of the compositions of the invention.

A method for treating or preventing an immune deficiency is provided according  
10 to another aspect of the invention. The method involves administering to a subject  
having or at risk of an immune deficiency, an effective amount for treating or preventing  
the immune deficiency of any of the compositions of the invention.

In another aspect the invention relates to a method for inducing a TH1 immune response by administering to a subject any of the compositions of the invention in an  
15 effective amount to produce a TH1 immune response.

In one embodiment the methods of the invention involve administering an oligonucleotide of formula 5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3' and an immunostimulatory nucleic acid having an unmethylated CG dinucleotide a TG dinucleotide or a T-rich sequence. In an embodiment the oligonucleotide comprising 5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3' is administered separately  
20 from the immunostimulatory nucleic acid. In some embodiments the oligonucleotide comprising 5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3' and the immunostimulatory nucleic acid are administered on an alternating weekly schedule and in other embodiments the oligonucleotide comprising 5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3' and the immunostimulatory nucleic acid are administered on an alternating biweekly schedule.

25       The invention provides in another aspect a composition, comprising an immunostimulatory nucleic acid and an anti-cancer therapy, formulated in a pharmaceutically-acceptable carrier and in an effective amount to treat a cancer or to reduce the risk of developing a cancer. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid.

The invention further provides a kit comprising a first container housing an immunostimulatory nucleic acid and at least one other container (e.g., a second

container) housing a an anti-cancer therapy, and instructions for use. In one embodiment, the kit further comprises interferon- $\alpha$ , which may be separately housed in yet another container (e.g., a third container). In an important embodiment, the kit comprises a sustained-release vehicle containing an immunostimulatory nucleic acid, and 5 at least one container housing an anti-cancer therapy, and instructions for timing of administration of the anti-cancer therapy. The immunostimulatory nucleic acid may be selected from the group consisting of a Py-rich nucleic acid, a TG nucleic acid and a CpG nucleic acid, wherein the CpG nucleic acid has a nucleotide sequence comprising SEQ ID NO: 246.

10 The invention further provides a method for preventing or treating asthma or allergy, comprising administering an immunostimulatory nucleic acid and an asthma/allergy medicament in an effective amount to treat or prevent the asthma or allergy. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid.

15 In one embodiment the immunostimulatory nucleic acid is a T-rich nucleic acid. In a related embodiment, the T-rich nucleic acid has a nucleotide sequence selected from the group consisting of SEQ ID NO: 59-63, 73-75, 142, 215, 226, 241, 267-269, 282, 301, 304, 330, 342, 358, 370-372, 393, 433, 471, 479, 486, 491, 497, 503, 556-558, 567, 694, 793-794, 797, 833, 852, 861, 867, 868, 882, 886, 905, 907, 908, and 910-913. In 20 other embodiments the T-rich nucleic acids are sequence selected from the group consisting of SEQ ID NO: 64, 98, 112, 146, 185, 204, 208, 214, 224, 233, 244, 246, 247, 258, 262, 263, 265, 270-273, 300, 305, 316, 317, 343, 344, 350, 352, 354, 374, 376, 392, 407, 411-413, 429-432, 434, 435, 443, 474, 475, 498-501, 518, 687, 692, 693, 804, 862, 883, 884, 888, 890, and 891.

25 In yet a further related embodiment, the T-rich nucleic acid is not a TG nucleic acid. In yet still another embodiment, the T-rich nucleic acid is not a CpG nucleic acid.

In one embodiment, the immunostimulatory nucleic acid is a TG nucleic acid. In a further related embodiment, the TG nucleic acid is not a T-rich nucleic acid. In another related embodiment, the TG nucleic acid is not a CpG nucleic acid.

30 In one embodiment, the immunostimulatory nucleic acid is a CpG nucleic acid, wherein the CpG nucleic acid has a nucleotide sequence comprising SEQ ID NO: 246.

- In another embodiment, the asthma/allergy medicament is a medicament selected from the group consisting of PDE-4 inhibitor, Bronchodilator/beta-2 agonist, K<sup>+</sup> channel opener, VLA-4 antagonist, Neurokinin antagonist, TXA2 synthesis inhibitor, Xanthanine, Arachidonic acid antagonist, 5 lipoxygenase inhibitor, Thromboxin A2 receptor antagonist, Thromboxane A2 antagonist, Inhibitor of 5-lipox activation protein, and Protease inhibitor, but is not so limited. In some important embodiments, the asthma/allergy medicament is a Bronchodilator/beta-2 agonist selected from the group consisting of salmeterol, salbutamol, terbutaline, D2522/formoterol, fenoterol, and orciprenaline.
- 10 In another embodiment, the asthma/allergy medicament is a medicament selected from the group consisting of Anti-histamines and Prostaglandin inducers. In one embodiment, the anti-histamine is selected from the group consisting of loratadine, cetirizine, buclizine, ceterizine analogues, fexofenadine, terfenadine, desloratadine, norastemizole, epinastine, ebastine, ebastine, astemizole, levocabastine, azelastine, 15 tranilast, terfenadine, mizolastine, betastastine, CS 560, and HSR 609. In another embodiment, the Prostaglandin inducer is S-5751.
- In yet another embodiment, the asthma/allergy medicament is selected from the group consisting of Steroids and Immunomodulators. The immunomodulators may be selected from the group consisting of anti-inflammatory agents, leukotriene antagonists, 20 IL4 muteins, Soluble IL-4 receptors, Immunosuppressants, anti-IL-4 antibodies, IL-4 antagonists, anti-IL-5 antibodies, soluble IL-13 receptor-Fc fusion proteins, anti-IL-9 antibodies, CCR3 antagonists, CCR5 antagonists, VLA-4 inhibitors, and Downregulators of IgE, but are not so limited. In one embodiment, the downregulator of IgE is an anti-IgE.
- 25 In another embodiment, the Steroid is selected from the group consisting of beclomethasone, fluticasone, tramcinolone, budesonide, and budesonide. In still a further embodiment, the Immunosuppressant is a Tolerizing peptide vaccine.
- In one embodiment, the immunostimulatory nucleic acid is administered concurrently with the asthma/allergy medicament. In another embodiment, the subject is 30 an immunocompromised subject

The immunostimulatory nucleic acids to be administered to a subject in the methods disclosed herein relating to the prevention and treatment of asthma/allergy are as described for other method aspects of the invention.

In another aspect, the invention provides a kit comprising a first container 5 housing an immunostimulatory nucleic acid, and at least another container (e.g., a second container) housing an asthma/allergy medicament, and instructions for use. The immunostimulatory nucleic acid useful in the kit is as described herein. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid. In another 10 important embodiment, the kit comprises a sustained-release vehicle containing an immunostimulatory nucleic acid, and at least one container housing an asthma/allergy medicament, and instructions for timing of administration of the asthma/allergy medicament. The asthma/allergy medicament may be selected from the group of asthma/allergy medicaments described in the foregoing methods directed towards the 15 prevention or treatment of asthma/allergy.

In yet another aspect, the invention provides a composition, comprising an immunostimulatory nucleic acid and an asthma/allergy medicament, formulated in a pharmaceutically-acceptable carrier and in an effective amount for preventing or treating an immune response associated with exposure to a mediator of asthma or allergy. The 20 immunostimulatory nucleic acid may be selected from the group of immunostimulatory nucleic acids described for the foregoing methods and compositions. In important embodiments, the immunostimulatory nucleic acid is selected from the group consisting of a T-rich nucleic acid, a TG nucleic acid and a C-rich nucleic acid. The asthma/allergy medicament may be selected from the group consisting of asthma medicaments and 25 allergy medicaments as described in the foregoing methods and compositions.

In still a further aspect, the invention provides a composition comprising an immunostimulatory nucleic acid selected from the group consisting of SEQ ID NO: 95-136, SEQ ID NO: 138-152, SEQ ID NO: 154-222, SEQ ID NO: 224-245, SEQ ID NO: 247-261, SEQ ID NO: 263-299, SEQ ID NO: 301, SEQ ID NO: 303-4109, SEQ ID NO: 30 414-420, SEQ ID NO: 424, SEQ ID NO: 426-947, SEQ ID NO: 959-1022, SEQ ID NO: 1024-1093, and a pharmaceutically acceptable carrier. Preferably the immunostimulatory nucleic acid is present in the composition in an effective amount. In

one embodiment, the immunostimulatory nucleic acid is present in an effective amount to induce an immune response. In another embodiment, the immunostimulatory nucleic acid is present in an effective amount to prevent or treat cancer. In yet a further embodiment, the immunostimulatory nucleic acid is present in an effective amount to prevent or treat asthma/allergy. The invention also provides kits comprising any of the foregoing immunostimulatory nucleic acid compositions, and instructions for use.

In another aspect the invention includes a composition of an immunostimulatory nucleic acid consisting essentially of: 5' M<sub>1</sub>TCGTCGTTM<sub>2</sub> 3' wherein at least one of the Cs is unmethylated, wherein M<sub>1</sub> is a nucleic acid having at least one nucleotide, wherein M<sub>2</sub> is a nucleic acid having between 0 and 50 nucleotides, and wherein the immunostimulatory nucleic acid has less than 100 nucleotides.

In yet other aspects the invention relates to a pharmaceutical composition of an immunostimulatory nucleic acid comprising: 5' TCGTCGTT 3' wherein at least one of the Cs is unmethylated, wherein the immunostimulatory nucleic acid has less than 100 nucleotides and a phosphodiester backbone, and a sustained release device. In some embodiments the sustained release device is a microparticle. In other embodiments the composition includes an antigen.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a histogram of the expression of CD86 (Y-axis) by CD19+ cells following exposure of these cells to the oligonucleotides shown on the X-axis at a concentration of 0.15 µg/ml.

Figure 1B is a histogram of the expression of CD86 (Y-axis) by CD19+ cells following exposure of these cells to the oligonucleotides shown on the X-axis at a concentration of 0.30 µg/ml.

Figure 2 is a graph comparing the abilities of ODN 2137, ODN 2177, ODN 2200 and ODN 2202 to stimulate B cell proliferation at concentrations ranging from 0.2 µg/ml to 20 µg/ml.

Figure 3 is a graph comparing the abilities of ODN 2188, ODN 2189, ODN 2190 and ODN 2182 to stimulate B cell proliferation at concentrations ranging from 0.2 µg/ml to 20 µg/ml.

Fig. 4 is a bar graph depicting dose-dependent B cell activation induced by non-CpG ODN. PBMC of a blood donor were incubated with the indicated concentrations of ODNs 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 10 5126 (SEQ ID NO.: 1058) and 5162 (SEQ ID NO.: 1094) and stained with mAb for CD19 (B cell marker) and CD86 (B cell activation marker, B7-2). Expression was measured by flow cytometry.

Fig. 5 is a bar graph depicting stimulation of B cells by a diverse set of non-CpG ODNs. PBMC of one representative donor were stimulated by 0.4µg/ml, 1.0µg/ml or 15 10.0µg/ml of the following ODNs: 2006 (SEQ ID NO.: 246), 2196 (SEQ ID NO.: 913), 2194 (SEQ ID NO.: 911), 5162 (SEQ ID NO.: 1094), 5163 (SEQ ID NO.: 1095), 5168 (SEQ ID NO.: 1096) and 5169 (SEQ ID NO.: 1097) and expression of the activation marker CD86 (B7-2) on CD19-positive B cells was measured by flow cytometry.

Fig 6 is a bar graph depicting B cell activation by non-CpG ODNs 1982 and 20 2041. PBMC were incubated with the indicated concentrations of ODN 2006 (SEQ ID NO.: 246), 1982 (SEQ ID NO.: 225) and 2041 (SEQ ID NO.: 282) and B cell activation (expression of the activation marker CD86) was measured by flow cytometry.

Fig. 7 is a bar graph depicting NK cells are activated by non-CpG ODNs. PBMC were incubated with 6µg/ml of the following ODNs: 2006 (SEQ ID NO.: 246), 2117 25 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058) and stained with mAb for CD3 (T cell marker), CD56 (NK cell marker) and CD69 (early activation marker). Expression of CD69 on CD56-positive NK cells was measured by flow cytometry.

Fig. 8 is a bar graph depicting NK-mediated cytotoxicity is enhanced by non-CpG ODN. NK-mediated lysis of K-562 target cells was measured after over night 30 incubation of PBMC with 6µg/ml of the ODN 2006 (SEQ ID NO.: 246), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058).

Fig. 9 is a bar graph depicting NKT cells can be activated by non-CpG ODN. PBMC of one representative donor were incubated with 6 $\mu$ g/ml ODN 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058) for 24h and activation of 5 NKT cells was measured by flow cytometry after staining of cells with mAb for CD3 (T cell marker), CD56 (NK cell marker) and CD69 (early activation marker).

Fig. 10 is a bar graph depicting stimulation of monocytes by different CpG and non-CpG ODN. PBMC were incubated with 6 $\mu$ g/ml 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2178 (SEQ ID NO.: 428), 2183 (SEQ ID 10 NO.: 433), 2194 (SEQ ID NO.: 911), 5126 (SEQ ID NO.: 1058) and 5163 (SEQ ID NO.: 1095) and stained for CD14 (monocyte marker) and CD80 (B7-1, activation marker). Expression was measured by flow cytometry.

Fig. 11 is a bar graph depicting release of TNF $\alpha$  upon culture of human cells with non-CpG ODN. PBMC were cultured for 24h with or without 6 $\mu$ g/ml of the indicated 15 ODNs or 1 $\mu$ g/ml LPS as positive control and TNF $\alpha$  measured by ELISA.

Fig. 12 is a bar graph depicting release of IL-6 after culture with non-CpG ODNs shows the same pattern as for TNF $\alpha$ . PBMC were cultured with the indicated ODNs (1.0 $\mu$ g/ml) and IL-6 was measured in the supernatants by ELISA.

20

#### DETAILED DESCRIPTION

The invention in one aspect involves the finding that pyrimidine (Py) rich and preferably thymidine (T) rich nucleic acids as well as nucleic acids that contain TG dinucleotide motifs are effective in mediating immune stimulatory effects. It was known in the prior art that CpG containing nucleic acids are therapeutic and prophylactic 25 compositions that stimulate the immune system to treat cancer, infectious diseases, allergy, asthma and other disorders and to help protect against opportunistic infections following cancer chemotherapies. The strong yet balanced, cellular and humoral immune responses that result from CpG stimulation reflect the body's own natural defense system against invading pathogens and cancerous cells. CpG sequences, while 30 relatively rare in human DNA are commonly found in the DNA of infectious organisms such as bacteria. The human immune system has apparently evolved to recognize CpG sequences as an early warning sign of infection, and to initiate an immediate and

powerful immune response against invading pathogens without causing adverse reactions frequently seen with other immune stimulatory agents. Thus CpG containing nucleic acids, relying on this innate immune defense mechanism, can utilize a unique and natural pathway for immune therapy. The effects of CpG nucleic acids on immune modulation

5 were discovered by the inventor of the instant patent application and have been described extensively in co-pending patent applications, such as U.S. Patent Application Serial Nos: 08/386,063 filed on 02/07/95 (and related PCT US95/01570); 08/738,652 filed on 10/30/96; 08/960,774 filed on 10/30/97 (and related PCT/US97/19791, WO 98/18810); 09/191,170 filed on 11/13/98; 09/030,701 filed on 02/25/98 (and related

10 PCT/US98/03678; 09/082,649 filed on 05/20/98 (and related PCT/US98/10408); 09/325,193 filed on 06/03/99 (and related PCT/US98/04703); 09/286,098 filed on 04/02/99 (and related PCT/US99/07335); 09/306,281 filed on 05/06/99 (and related PCT/US99/09863). The entire contents of each of these patents and patent applications is hereby incorporated by reference.

15 The findings of the instant invention are applicable to all of the above described uses of CpG containing nucleic acids as well as any other known use for CpG nucleic acids. The invention involves, in one aspect, the discovery that Py-rich and preferably T-rich and TG nucleic acids have similar immune stimulatory properties to CpG oligonucleotides regardless of whether a CpG motif is present. Thus the invention is

20 useful for any method for stimulating the immune system using Py-rich or TG nucleic acids. It was also discovered surprisingly according to the invention that chimeric oligonucleotides which lack a CpG motif are immune stimulatory and have many of the same prophylactic and therapeutic activities as a CpG oligonucleotide.

A Py-rich nucleic acid is a T-rich or C-rich immunostimulatory nucleic acid. In

25 some embodiments T-rich nucleic acids are preferred. A T-rich nucleic acid is a nucleic acid which includes at least one poly T sequence and/or which has a nucleotide composition of greater than 25% T nucleotide residues. A nucleic acid having a poly-T sequence includes at least four Ts in a row, such as 5'TTTT3'. Preferably the T-rich nucleic acid includes more than one poly T sequence. In preferred embodiments the T-rich nucleic acid may have 2, 3, 4, etc poly T sequences, such as oligonucleotide #2006

30 (SEQ ID NO:246). One of the most highly immunostimulatory T-rich oligonucleotides discovered according to the invention is a nucleic acid composed entirely of T nucleotide

residues, e.g., oligonucleotide #2183 (SEQ ID NO:433). Other T-rich nucleic acids according to the invention have a nucleotide composition of greater than 25% T nucleotide residues, but do not necessarily include a poly T sequence. In these T-rich nucleic acids the T nucleotide resides may be separated from one another by other types 5 of nucleotide residues, i.e., G, C, and A. In some embodiments the T-rich nucleic acids have a nucleotide composition of greater than 35%, 40%, 50%, 60%, 70%, 80%, 90%, and 99%, T nucleotide residues and every integer % in between. Preferably the T-rich nucleic acids have at least one poly T sequence and a nucleotide composition of greater than 25% T nucleotide residues.

10 It was discovered according to the invention that the T content of an ODN has a dramatic effect on the immune stimulatory effect of the ODN and that T-rich ODN can activate multiple human immune cell types in the absence of any CpG motifs. An oligonucleotide having a 3' poly-T region and 2 5'CGs e.g., ODN 2181 (SEQ ID NO:431) is highly immune stimulatory. An oligonucleotide of similar length, ODN 15 2116 (SEQ ID NO:357) which contains two CG dinucleotides at the 5' end and a poly-C region at the 3' end was also immune stimulatory but to a lesser extent than the T-rich oligonucleotide using standard experimental conditions. Thus, although C and T have almost identical structures, their effects on the immune properties of an ODN are varied. They both are capable of inducing an immune response but to different extents. Thus 20 both T-rich and C-rich oligonucleotides are useful according to the invention, but T-rich oligonucleotides are preferred. Furthermore, if the T content of the ODN is reduced by incorporating other bases such as G, A, or C, then the immune stimulatory effects are reduced (ODN #2188 (SEQ ID NO:905), 2190 (SEQ ID NO:907), 2191 (SEQ ID NO:908), and 2193 (SEQ ID NO:910)).

25 A C-rich nucleic acid is a nucleic acid molecule having at least one or preferably at least two poly-C regions or which is composed of at least 50% C nucleotides. A poly-C region is at least four C residues in a row. Thus a poly-C region is encompassed by the formula 5'CCCC 3'. In some embodiments it is preferred that the poly-C region have the formula 5'CCCCCC 3'. Other C-rich nucleic acids according to the invention 30 have a nucleotide composition of greater than 50% C nucleotide residues, but do not necessarily include a poly C sequence. In these C-rich nucleic acids the C nucleotide residues may be separated from one another by other types of nucleotide residues, i.e., G,

T, and A. In some embodiments the C-rich nucleic acids have a nucleotide composition of greater than 60%, 70%, 80%, 90%, and 99%, C nucleotide residues and every integer % in between. Preferably the C-rich nucleic acids have at least one poly C sequence and a nucleotide composition of greater than 50% C nucleotide residues, and in some 5 embodiments are also T-rich.

As shown in the Examples, several ODN previously believed to be non-immunostimulatory, including two ODNs SEQ ID NO.: 225 and SEQ ID NO.: 282 previously described to be non-stimulatory and mainly used as control ODNs (Takahashi, T., M. Nieda, Y. Koezuka, A. Nicol, S. A. Porcelli, Y. Ishikawa, K. 10 Tadokoro, H. Hirai, and T. Juji. 2000. Analysis of human VA24+ CD4+ NKT cells activated by  $\alpha$ -glycosylceramide-pulsed monocyte-derived dendritic cells. *J. Immunol.* 164:4458) were found to be immunostimulatory. Our experiments, demonstrated that these ODNs can stimulate B cells, although at higher concentrations compared to CpG 15 ODNs (Fig. 6). A long Poly T ODN (30mer) induced, at least in some experiments, comparable strong activation of B cells to one of the strongest CpG ODN activators of B cells. These experiments also revealed the surprising finding that even Poly C ODNs can lead to stimulation of B cells.

Immunostimulation by these ODNs, however, was not limited to human B cells. Different experimental assays clearly demonstrated in addition that monocytes, NK cells 20 and even NKT cells can be activated by such non-CpG ODNs (Fig. 7 – 10). In contrast to Poly T and Poly C sequences, immunostimulation by Poly A sequences (at least for monocytes, B and NK cells) was not achieved. Interestingly it was found that the introduction of a CpG motif into SEQ ID NO.: 225 enhanced the immunostimulatory activity whereas the elongation with a Poly T stretch did not enhance 25 immunostimulation. This suggests that CpG and T-rich ODN may operate through different mechanisms or pathways. It is also possible that insertion of a poly-T motif into a different position of SEQ ID NO.: 225 may result in a change in immunostimulatory properties.

A “TG nucleic acid” or a “TG immunostimulatory nucleic acid” as used herein is 30 a nucleic acid containing at least one TpG dinucleotide (thymidine-guanine dinucleotide sequence, i.e. “TG DNA” or DNA containing a 5' thymidine followed by 3' guanosine and linked by a phosphate bond) and activates a component of the immune system.

In one embodiment the invention provides a TG nucleic acid represented by at least the formula:



wherein  $X_1$  and  $X_2$  are nucleotides and  $N$  is any nucleotide and  $N_1$  and  $N_2$  are 5 nucleic acid sequences composed of any number of  $N$  provided that the sum total of  $N_1$  and  $N_2$  is in the range of 11 to 21. As an example, if  $N_1$  is 5, then  $N_2$  may be 6 (leading to a total length for the oligonucleotide of 15 nucleotides). The TG may be located anywhere within the oligonucleotide stretch, including the 5' end, the center and the 3' end. Thus,  $N_1$  may be zero through to 21, inclusive, provided that  $N_2$  is appropriately 10 chosen to give a sum of  $N_2$  and  $N_1$  equal to 11 through to 21, inclusive. Similarly,  $N_2$  may be zero through to 21, inclusive, provided that the sum total of  $N_1$  and  $N_2$  equals 11 to 21, inclusive. In some embodiments  $X_1$  is adenine, guanine, or thymidine and  $X_2$  is cytosine, adenine, or thymidine. In one preferred embodiment,  $X_2$  is thymidine. In other 15 embodiments  $X_1$  is cytosine and/or  $X_2$  is guanine. In other embodiments, as discussed herein, the nucleic acid may encompass other motifs, provided it is long enough to do so.

In other embodiments the TG nucleic acid is represented by at least the formula:



wherein  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  are nucleotides. In some embodiments,  $X_1X_2$  are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, 20 CpT, CpA, TpA and TpT; and  $X_3X_4$  are nucleotides selected from the group consisting of: TpT, CpT, ApT, ApG, TpC, ApC, CpC, TpA, ApA, and CpA;  $N$  is any nucleotide and  $N_1$  and  $N_2$  are nucleic acid sequences composed of any number of nucleotides provide that the sum total of  $N_1$  and  $N_2$  is in the range of 9 to 19. In some embodiments, 25  $X_1X_2$  are GpA or GpT and  $X_3X_4$  are TpT. In other embodiments  $X_1$  or  $X_2$  or both are purines and  $X_3$  or  $X_4$  or both are pyrimidines or  $X_1X_2$  are GpA and  $X_3$  or  $X_4$  or both are pyrimidines. In one preferred embodiment,  $X_3X_4$  are nucleotides selected from the group consisting of: TpT, TpC and TpA.

The immunostimulatory nucleic acid may be any size (i.e., length) provided it is at least 4 nucleotides. In important embodiments, the immunostimulatory nucleic acids 30 have a length in the range of between 6 and 100. In still other embodiments, the length is in the range of between 8 and 35 nucleotides. Preferably, the TG oligonucleotides range in size from 15 to 25 nucleotides.

The size (i.e., the number of nucleotide residues along the length of the nucleic acid) of the immunostimulatory nucleic acid may also contribute to the stimulatory activity of the nucleic acid. It has been discovered, surprisingly that even for highly immune stimulating immunostimulatory nucleic acids, the length of the nucleic acid

5 influences the extent of immunostimulation that can be achieved. It has been demonstrated that increasing the length of a T-rich nucleic acid up to 24 nucleotides causes increased immune stimulation. The experiments presented in the examples demonstrate that when the length of the T-rich nucleic acid is increased from 18 to 27 nucleotides the ability of the nucleic acid to stimulate an immune response is increased

10 significantly (compare ODN #2194, 2183, 2195, and 2196 decreasing in size from 27-18 nucleotides). Increasing the length of the nucleic acid up to 30 nucleotides had a dramatic impact on the biological properties of the nucleic acid but increasing the length beyond 30 nucleotides did not appear to further influence the immune stimulatory effect (e.g., compare ODN 2179 to 2006).

15 It has been shown that TG nucleic acids ranging in length from 15 to 25 nucleotides in length may exhibit an increased immune stimulation. Thus, in one aspect, the invention provides an oligonucleotide that is 15-27 nucleotides in length (i.e., an oligonucleotide that is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 nucleotides in length) that may be a T-rich nucleic acid or may be a TG nucleic acid, or may be both a

20 T-rich and a TG nucleic acid. In one embodiment, the oligonucleotide is not a T-rich nucleic acid nor is it a TG nucleic acid. In other embodiments, the oligonucleotide does not have a CG motif. The invention similarly provides oligonucleotides that are 15-27 nucleotides in length, oligonucleotides that are 18-25 nucleotides in length, oligonucleotides that are 20-23 nucleotides in length, and oligonucleotides that are 23-

25 25 nucleotides in length. Any of the foregoing embodiments relating to oligonucleotides 15-27 in length also relate to the oligonucleotides of these differing lengths. The invention further embraces the use of any of these foregoing oligonucleotides in the methods recited herein.

Although a maximal level of immune stimulation is achieved with some T-rich nucleic acids when the nucleic acid is 24-30 nucleotide residues in length, as well as with some TG nucleic acids that range from 15 to 25 nucleotides in length, shorter or longer immunostimulatory nucleic acids can also be used according to the methods of the

invention. For facilitating uptake into cells immunostimulatory nucleic acids preferably have a minimum length of 6 nucleotide residues. Nucleic acids of any size greater than 6 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded inside of cells. Preferably the immunostimulatory nucleic acids are in the range of between 8 and 100 and in some embodiments T-rich containing immunostimulatory nucleic acids are between 24 and 40 nucleotides in length and TG containing immunostimulatory nucleic acids are between 15 and 25 nucleotides in length.

- 10 In one embodiment the T-rich nucleic acid is represented by at least the formula:



wherein  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  are nucleotides. In one embodiment  $X_1X_2$  is TT and/or  $X_3X_4$  is TT. In another embodiment  $X_1X_2$  are any one of the following nucleotides TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, GT, GG, GA, and GC; and  $X_3X_4$  are any 15 one of the following nucleotides TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, GT, GG, GA, and GC.

In some embodiments it is preferred that the immunostimulatory nucleic acids do not contain poly-C (CCCC), or poly-A (AAAA). In other embodiments it is preferred that the immunostimulatory nucleic acid include poly-C, poly-A, poly-G (GGGG) or multiple GGs. In particular poly-G or multiple GG motifs have dramatic effects on some immunostimulatory nucleic acids. The effect of these non-T sequences depends in part 20 on the status of the nucleic acid backbone. For instance, if the nucleic acid has a phosphodiester backbone or a chimeric backbone the inclusion of these sequences in the nucleic acid will only have minimal if any effect on the biological activity of the nucleic acid. If the backbone is completely phosphorothioate (or other phosphate modification) 25 or significantly phosphorothioate then the inclusion of these sequences may have more influence on the biological activity or the kinetics of the biological activity, causing a decrease in potency of the T-rich and TG immunostimulatory nucleic acids.

Although C-rich nucleic acids have been demonstrated to have immune stimulating properties, insertion of Poly-C sequences into a T-rich nucleic acid in a manner that would reduce the relative proportion of T nucleotides in the nucleic acid can have a negative impact on the nucleic acid. Although applicants are not bound by a

proposed mechanism, it is believed that the immune system has developed a mechanism for distinguishing nucleic acids having different nucleotide properties, possibly resulting from different sets of binding proteins which recognize different sequences or specific binding proteins which recognize all the immunostimulatory sequences but with different affinities. In general nucleic acids including unmethylated CpG motifs are the most immunostimulatory, followed by T-rich nucleic acids, TG nucleic acids and C-rich nucleic acids. This generalization, however, has many exceptions. For instance a strong T-rich nucleic acid like SEQ ID NO.: 886 is more immune stimulatory in some assays than some CpG containing nucleic acids (e.g., a phosphorothioate CpG nucleic acid containing a single CpG motif).

It has also been discovered that the addition of a poly-A tail to an immunostimulatory nucleic acid can enhance the activity of the nucleic acid. It was discovered that when a highly immune stimulatory CpG nucleic acid (SEQ ID NO.: 246) was modified with the addition of a poly-A tail (AAAAAA) or a poly-T tail (TTTTT), the resultant oligonucleotides increased in immune stimulatory activity. The ability of the poly-A tail and the poly-T tail to increase the immunostimulating properties of the oligonucleotide was very similar. SEQ ID NO.: 246 is a T-rich oligonucleotide. It is likely that if poly-A and poly-T tails are added to a nucleic acid which is not T-rich, it would have a bigger impact on the immune stimulating capability of the nucleic acid.

Since the poly-T tail was added to a nucleic acid that was already highly T-rich the immune stimulating properties of the poly-T addition was diluted somewhat, although not completely. This finding has important implications for the use of poly-A regions. Thus in some embodiments the immunostimulatory nucleic acids include a poly-A region and in other embodiments they do not.

Some of the immunostimulatory nucleic acids of the invention include one or more CG motifs. The presence of CG motifs in the immunostimulatory nucleic acids also has an influence on the biological activity of the nucleic acids. If the total length of an immunostimulatory nucleic acid is 20 nucleotide residues or less, then CpG motifs are important in determining the immune effect of the nucleic acid, and methylation of these motifs reduces the potency of the immune stimulatory effects of the nucleic acid. If the length of the immunostimulatory nucleic acid is increased to 24, then the immune stimulatory effects of the nucleic acid become less dependent on the CpG motifs, and are

no longer abolished by methylation of the CpG motifs or by their inversion to GC dinucleotides, provided the other immune-stimulatory properties described herein are present.

For example, ODN 2006 (SEQ ID NO:246) is a highly immune stimulatory T-rich nucleic acid of 24 nucleotide residues in length with four CpG dinucleotides. However, ODN 2117 (SEQ ID NO:358), in which the CpG motifs are methylated is also highly immune stimulatory. ODN 2137 (SEQ ID NO:886), in which the CpG motifs of ODN 2006 are inverted to GpC, and which as a result possesses six TG dinucleotides is also immune stimulatory. The immune stimulatory effects of nucleic acids such as ODN 2117 and 2137 are regulated by their T and TG content. Each of these three nucleic acids is T-rich and ODN 2137 is additionally TG rich. If their T content is reduced by inserting other bases such as A (ODN 2117 (SEQ ID NO:358)) or if their TG content is reduced by substituting TG with AG, then the immune stimulatory effects are somewhat reduced. In another example, a nucleic acid 24 nucleotides in length in which all of the positions are randomized has only a modest immune stimulatory effect (ODN 2182 (SEQ ID NO:432)). Likewise, a nucleic acid 24 nucleotides in length with other nucleotide compositions have variable immune stimulatory effects, depending on their T content (ODN 2188 (SEQ ID NO:905), 2189 (SEQ ID NO:906), 2190 (SEQ ID NO:907), 2191 (SEQ ID NO:908), 2193 (SEQ ID NO:910), 2183 (SEQ ID NO:433), and 2178 (SEQ ID NO:428)). ODN 2190 which contains TGT motifs is more immune stimulatory than ODN 2202 which possesses TGG motifs. Thus, in some embodiments, TGT motifs are preferred. In still other embodiments, the number of TG motifs is important in that an increase in the number of TG motifs leads to an increase in immune stimulation. Some preferred TG nucleic acids contain at least three TG motifs.

Examples of CpG nucleic acids include but are not limited to those listed in Table A, such as SEQ ID NO: 1, 3, 4, 14-16, 18-24, 28, 29, 33-46, 49, 50, 52-56, 58, 64-67, 69, 71, 72, 76-87, 90, 91, 93, 94, 96, 98, 102-124, 126-128, 131-133, 136-141, 146-150, 152-153, 155-171, 173-178, 180-186, 188-198, 201, 203-214, 216-220, 223, 224, 227-240, 242-256, 258, 260-265, 270-273, 275, 277-281, 286-287, 292, 295-296, 300, 302, 305-307, 309-312, 314-317, 320-327, 329, 335, 337-341, 343-352, 354, 357, 361-365, 367-369, 373-376, 378-385, 388-392, 394, 395, 399, 401-404, 406-426, 429-433, 434-437, 439, 441-443, 445, 447, 448, 450, 453-456, 460-464, 466-469, 472-475, 477, 478,

480, 483-485, 488, 489, 492, 493, 495-502, 504-505, 507-509, 511, 513-529, 532-541,  
543-555, 564-566, 568-576, 578, 580, 599, 601-605, 607-611, 613-615, 617, 619-622,  
625-646, 648-650, 653-664, 666-697, 699-706, 708, 709, 711-716, 718-732, 736, 737,  
739-744, 746, 747, 749-761, 763, 766-767, 769, 772-779, 781-783, 785-786, 7900792,  
5 798-799, 804-808, 810, 815, 817, 818, 820-832, 835-846, 849-850, 855-859, 862, 865,  
872, 874-877, 879-881, 883-885, 888-904, and 909-913.

In some embodiments of the invention the immunostimulatory nucleic acids include CpG dinucleotides and in other embodiments the immunostimulatory nucleic acids are free of CpG dinucleotides. The CpG dinucleotides may be methylated or unmethylated. A nucleic acid containing at least one unmethylated CpG dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing an unmethylated 5' cytosine followed by 3' guanosine and linked by a phosphate bond) and activates the immune system. A nucleic acid containing at least one methylated CpG dinucleotide is a nucleic acid which 10 contains a methylated cytosine-guanine dinucleotide sequence (i.e., a methylated 5' cytosine followed by a 3' guanosine and linked by a phosphate bond).  
15

Examples of T rich nucleic acids that are free of CpG nucleic acids include but are not limited to those listed in Table A, such as SEQ ID NO: 59-63, 73-75, 142, 215, 226, 241, 267-269, 282, 301, 304, 330, 342, 358, 370-372, 393, 433, 471, 479, 486, 491, 20 497, 503, 556-558, 567, 694, 793-794, 797, 833, 852, 861, 867, 868, 882, 886, 905, 907, 908, and 910-913. Examples of T rich nucleic acids that include CpG nucleic acids include but are not limited to those listed in Table A, such as SEQ ID NO: 64, 98, 112, 146, 185, 204, 208, 214, 224, 233, 244, 246, 247, 258, 262, 263, 265, 270-273, 300, 305, 316, 317, 343, 344, 350, 352, 354, 374, 376, 392, 407, 411-413, 429-432, 434, 435, 443, 25 474, 475, 498-501, 518, 687, 692, 693, 804, 862, 883, 884, 888, 890, and 891.

The immunostimulatory nucleic acids can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased immune activity. Thus in some aspects of the invention it is preferred that the nucleic acid be single stranded and in other aspects it is preferred that the nucleic acid be double stranded.  
30

The term T-rich nucleic acid and TG nucleic acid, as used herein, refers to an immunostimulatory T-rich nucleic acid and an immunostimulatory TG nucleic acid,

respectively, unless otherwise indicated. The T-rich nucleic acid sequences of the invention are those broadly described above as well as the nucleic acids shown in Table A that have at least one poly T motif and/or have a composition of greater than 25% T or preferably 35% nucleotide residues. The C-rich nucleic acids are those having at least 5 one and preferably at least two poly-C regions. The TG nucleic acids of the invention are those broadly described above as well as the specific nucleic acids shown in Table A that have at least one TG motif.

The nucleic acids of the invention may, but need not, also include a poly G motif. Poly G containing nucleic acids are also immunostimulatory. A variety of references, 10 including Pisetsky and Reich, 1993 *Mol. Biol. Reports*, 18:217-221; Krieger and Herz, 1994, *Ann. Rev. Biochem.*, 63:601-637; Macaya et al., 1993, *PNAS*, 90:3745-3749; Wyatt et al., 1994, *PNAS*, 91:1356-1360; Rando and Hogan, 1998, In Applied Antisense Oligonucleotide Technology, ed. Krieg and Stein, p. 335-352; and Kimura et al., 1994, *J. Biochem.* 116, 991-994 also describe the immunostimulatory properties of poly G 15 nucleic acids.

Poly G nucleic acids preferably are nucleic acids having the following formulas:



wherein  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  are nucleotides. In preferred embodiments at least one of  $X_3$  and  $X_4$  are a G. In other embodiments both of  $X_3$  and  $X_4$  are a G. In yet other 20 embodiments the preferred formula is 5' GGGNGGG 3', or 5' GGGNGGGNGGG 3' wherein N represents between 0 and 20 nucleotides. In other embodiments the poly G nucleic acid is free of unmethylated CG dinucleotides, such as, for example, the nucleic acids listed below as SEQ ID NO: 5, 6, 73, 215, 267-269, 276, 282, 288, 297-299, 355, 359, 386, 387, 444, 476, 531, 557-559, 733, 768, 795, 796, 914-925, 928-931, 933-936, 25 and 938. In other embodiments the poly G nucleic acid includes at least one unmethylated CG dinucleotide, such as, for example, the nucleic acids listed above as SEQ ID NO: 67, 80-82, 141, 147, 148, 173, 178, 183, 185, 214, 224, 264, 265, 315, 329, 434, 435, 475, 519, 521-524, 526, 527, 535, 554, 565, 609, 628, 660, 661, 662, 725, 767, 825, 856, 857, 876, 892, 909, 926, 927, 932, and 937.

30 The terms "nucleic acid" and "oligonucleotide" are used interchangeably to mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a

substituted pyrimidine (e.g. cytosine (C), thymidine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). As used herein, the terms refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms shall also include polynucleosides (i.e. a polynucleotide minus the phosphate) and any other organic base  
5 containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g. produced by nucleic acid synthesis).

- The terms nucleic acid and oligonucleotide also encompass nucleic acids or oligonucleotides with substitutions or modifications, such as in the bases and/or sugars.  
10 For example, they include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose instead of ribose. Thus the nucleic acids may be  
15 heterogeneous in backbone composition thereby containing any possible combination of polymer units linked together such as peptide- nucleic acids (which have amino acid backbone with nucleic acid bases). In some embodiments, the nucleic acids are homogeneous in backbone composition. Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne modified bases (Wagner et al., *Nature Biotechnology* 14:840- 844, 1996). Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymidine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties.  
20 Other such modifications are well known to those of skill in the art.  
25 For use in the instant invention, the nucleic acids of the invention can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg et al., *Tet. Let.* 27:4051-4054, 1986; Froehler et al., *Nucl. Acid. Res.* 14:5399-5407, 1986, ; Garegg et al., *Tet. Let.* 27:4055-4058, 1986, Gaffney et al., *Tet. Let.* 29:2619-2622, 1988).  
30 These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These nucleic acids are referred to as synthetic nucleic acids.

Alternatively, T-rich and/or TG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor laboratory Press, New York, 1989) and separated into smaller pieces or administered whole. Nucleic acids can be prepared from existing nucleic acid sequences 5 (*e.g.*, genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases. Nucleic acids prepared in this manner are referred to as isolated nucleic acid. An isolated nucleic acid generally refers to a nucleic acid which is separated from components which it is normally associated with in nature. As an example, an isolated nucleic acid may be one which is separated from a cell, from 10 a nucleus, from mitochondria or from chromatin. The terms Py-rich nucleic acids and TG nucleic acids encompasses both synthetic and isolated Py-rich nucleic acids and TG nucleic acids.

For use *in vivo*, the Py-rich and TG nucleic acids may optionally be relatively resistant to degradation (*e.g.*, are stabilized). A "stabilized nucleic acid molecule" shall 15 mean a nucleic acid molecule that is relatively resistant to *in vivo* degradation (*e.g.* via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Nucleic acids that are tens to hundreds of kbs long are relatively resistant to *in vivo* degradation. For shorter nucleic acids, secondary structure can stabilize and increase 20 their effect. For example, if the 3' end of an nucleic acid has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop structure, then the nucleic acid becomes stabilized and therefore exhibits more activity.

Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. Preferred stabilized nucleic acids of the instant invention have a modified backbone. It has been demonstrated that modification of the nucleic acid 25 backbone provides enhanced activity of the Py-rich and TG nucleic acids when administered *in vivo*. These stabilized structures are preferred because the Py-rich and TG molecules of the invention have at least a partial modified backbone. Py-rich and TG constructs having phosphorothioate linkages provide maximal activity and protect the nucleic acid from degradation by intracellular exo- and endo-nucleases. Other 30 modified nucleic acids include phosphodiester modified nucleic acids, combinations of phosphodiester and phosphorothioate nucleic acid, methylphosphonate, methylphosphorothioate, phosphorodithioate, p-ethoxy, and combinations thereof. Each

of these combinations and their particular effects on immune cells is discussed in more detail with respect to CpG nucleic acids in PCT Published Patent Applications PCT/US95/01570 (WO 96/02555) and PCT/US97/19791 (WO 98/18810) claiming priority to U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and 5 October 30, 1997 respectively, the entire contents of which are hereby incorporated by reference. It is believed that these modified nucleic acids may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

The compositions of the invention may optionally be chimeric oligonucleotides. 10 The chimeric oligonucleotides are oligonucleotides having a formula: 5' Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> 3'. Y<sub>1</sub> and Y<sub>2</sub> are nucleic acid molecules having between 1 and 10 nucleotides. Y<sub>1</sub> and Y<sub>2</sub> each include at least one modified internucleotide linkage. Since at least 2 nucleotides of the chimeric oligonucleotides include backbone modifications these nucleic acids are an example of one type of "stabilized immunostimulatory nucleic acids."

15 With respect to the chimeric oligonucleotides, Y<sub>1</sub> and Y<sub>2</sub> are considered independent of one another. This means that each of Y<sub>1</sub> and Y<sub>2</sub> may or may not have different sequences and different backbone linkages from one another in the same molecule. The sequences vary, but in some cases Y<sub>1</sub> and Y<sub>2</sub> have a poly-G sequence. A poly-G sequence refers to at least 3 Gs in a row. In other embodiments the poly-G 20 sequence refers to at least 4, 5, 6, 7, or 8 Gs in a row. In other embodiments Y<sub>1</sub> and Y<sub>2</sub> may be TCGTCG, TCGTCGT, or TCGTCGTT (SEQ ID NO:1145). Y<sub>1</sub> and Y<sub>2</sub> may also have a poly-C, poly-T, or poly-A sequence. In some embodiments Y<sub>1</sub> and/or Y<sub>2</sub> have between 3 and 8 nucleotides.

25 N<sub>1</sub> and N<sub>2</sub> are nucleic acid molecules having between 0 and 5 nucleotides as long as N<sub>1</sub>ZN<sub>2</sub> has at least 6 nucleotides in total. The nucleotides of N<sub>1</sub>ZN<sub>2</sub> have a phosphodiester backbone and do not include nucleic acids having a modified backbone.

30 Z is an immunostimulatory nucleic acid motif but does not include a CG. For instance, Z may be a nucleic acid a T-rich sequence, e.g. including a TTTT motif or a sequence wherein at least 50% of the bases of the sequence are Ts or Z may be a TG sequence.

The center nucleotides (N<sub>1</sub>ZN<sub>2</sub>) of the formula Y<sub>1</sub>N<sub>1</sub>ZN<sub>2</sub>Y<sub>2</sub> have phosphodiester internucleotide linkages and Y<sub>1</sub> and Y<sub>2</sub> have at least one, but may have more than one or

even may have all modified internucleotide linkages. In preferred embodiments Y<sub>1</sub> and/or Y<sub>2</sub> have at least two or between two and five modified internucleotide linkages or Y<sub>1</sub> has two modified internucleotide linkages and Y<sub>2</sub> has five modified internucleotide linkages or Y<sub>1</sub> has five modified internucleotide linkages and Y<sub>2</sub> has two modified internucleotide linkages. The modified internucleotide linkage, in some embodiments is a phosphorothioate modified linkage, a phosphorodithioate modified linkage or a p-ethoxy modified linkage.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., *Chem. Rev.* 90:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

Other stabilized nucleic acids include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acids which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

In the case when the Py-rich or TG nucleic acid is administered in conjunction with an antigen which is encoded in a nucleic acid vector, it is preferred that the backbone of the Py-rich or TG nucleic acid be a chimeric combination of phosphodiester and phosphorothioate (or other phosphate modification). The cell may have a problem taking up a plasmid vector in the presence of completely phosphorothioate nucleic acid. Thus when both a vector and a nucleic acid are delivered to a subject, it is preferred that the nucleic acid have a chimeric backbone or have a phosphorothioate backbone but that the plasmid be associated with a vehicle that delivers it directly into the cell, thus avoiding the need for cellular uptake. Such vehicles are known in the art and include, for example, liposomes and gene guns.

The nucleic acids described herein as well as various control nucleic acids are presented below in Table A.

Table A

| SEQ ID NO: | ODN SEQUENCE              | BACKBONE |
|------------|---------------------------|----------|
| 1          | tctcccacgcgtgcgcacat      | s        |
| 2          | ataatccacgttgaaccaag      | s        |
| 3          | ataatcgacgttcaagcaag      | s        |
| 4          | taccggcgtgcgaccctct       | s        |
| 5          | ggggagggt                 | s        |
| 6          | ggggagggg                 | s        |
| 7          | ggtgagggt                 | s        |
| 8          | tccatgtzgttcctgatgct      | o        |
| 9          | gctacaccttagzgtga         | o        |
| 10         | tccatgazgttcctgatgct      | o        |
| 11         | tccatgacgttcztgatgct      | o        |
| 12         | gctagazgttagtgt           | o        |
| 13         | agctccatgtgtcactg         | s        |
| 14         | ccacgtcgaccctcaggcga      | s        |
| 15         | gcacatcgccccgcagccga      | s        |
| 16         | gtcaactcggttacctcga       | s        |
| 17         | gttggatacaggccagacttttttg | o        |
| 18         | gattcaacttgcgtcatcttaggc  | o        |
| 19         | accatggacgaactgttccccctc  | s        |
| 20         | accatggacgagctgttccccctc  | s        |
| 21         | accatggacgacctgttccccctc  | s        |
| 22         | accatggacgtactgttccccctc  | s        |
| 23         | accatggacggctgttccccctc   | s        |
| 24         | accatggacgttctgttccccctc  | s        |
| 25         | ccactcacatctgtgtccacaag   | o        |
| 26         | acttctcatagtcccttggtccag  | o        |
| 27         | tccatgagcttcctgagtc       | o        |
| 28         | gaggaaggigiggaigacgt      | o        |
| 29         | gtgaaticgttccgtggict      | o        |
| 30         | aaaaaa                    | s        |
| 31         | cccccc                    | s        |
| 32         | ctgtca                    | s        |
| 33         | tcgttag                   | s        |
| 34         | tcgtgg                    | s        |
| 35         | cgtcgt                    | s        |
| 36         | tccatgtcggtcctgagtc       | sos      |
| 37         | tccatgccgtcctgagtc        | sos      |
| 38         | tccatgacggtcctgagtc       | sos      |
| 39         | tccatgacgttcctgagtc       | sos      |
| 40         | tccatgtcgatcctgagtc       | sos      |
| 41         | tccatgtcgctcctgagtc       | sos      |
| 42         | tccatgtcggtcctgagtc       | sos      |
| 43         | tccatgacgttcctgagtc       | sos      |

|    |                                       |     |
|----|---------------------------------------|-----|
| 44 | tccataacgttcctgagtc                   | sos |
| 45 | tccatgacgtccctgagtc                   | sos |
| 46 | tccatcacgtgcctgagtc                   | sos |
| 47 | tccatgtggtcctgagtc                    | sos |
| 48 | tccatgtzggtcctgagtc                   | sos |
| 49 | ccgcttcctccagatgagtcatgggttctccaccaag | o   |
| 50 | cttggtgagaaaaccatgagtcatctggaggaagcgg | o   |
| 51 | ccccaaagggtatgagaagt                  | o   |
| 52 | agatagcaaatcggtgacg                   | o   |
| 53 | ggttcacgtgctcatggctg                  | o   |
| 54 | tctcccagcgtgcgcatt                    | s   |
| 55 | tctcccagcgtgcgcatt                    | s   |
| 56 | taccgcgtgcgcatt                       | s   |
| 57 | ataatccagttgaaccaag                   | s   |
| 58 | ataatcgacgttcaagcaag                  | s   |
| 59 | tccatgattttcctgatTTT                  | o   |
| 60 | ttgttttttttttttttttttt                | s   |
| 61 | tttttttttttttttttttt                  | o   |
| 62 | tgtgtctttttgtgttttttt                 | s   |
| 63 | tgtgtcttgcgtttttgtctt                 | o   |
| 64 | gcattcatcaggcgcccgaagaat              | o   |
| 65 | taccgagcttcgacgagattca                | o   |
| 66 | gcatgacgtttagct                       | s   |
| 67 | cacgtttaggggcat                       | s   |
| 68 | ctgctgagactggag                       | s   |
| 69 | tccatgacgttcctgacgtt                  | s   |
| 70 | gcatgagctttagctga                     | o   |
| 71 | tcagcgtgcgc                           | s   |
| 72 | atgacgttcctgacgtt                     | s   |
| 73 | tttggggtttggggttt                     | s   |
| 74 | tctaggcttttaggtttcc                   | s   |
| 75 | tgcatttttaggccaccat                   | s   |
| 76 | tctcccagcgtgcgtgcgcatt                | s   |
| 77 | tctcccagcgccat                        | s   |
| 78 | tctcccagcgtgcgcatt                    | s   |
| 79 | tctcccagcgcgcgcatt                    | s   |
| 80 | ggggtagcgttcagggggg                   | sos |
| 81 | ggggtagcgtgcgcattgggg                 | sos |
| 82 | ggggtagcgttcagggggg                   | sos |
| 83 | tccatgtcgttcctgtctt                   | s   |
| 84 | tccatagcgttcctagcgtt                  | s   |
| 85 | tctcgctgtctccgttctt                   | s   |
| 86 | gcatgacgtttagct                       | sos |
| 87 | tctcccagcgtgcgcattat                  | sos |
| 88 | tccatgazgttcctgazgtt                  | s   |
| 89 | gcatgazgtttagct                       | o   |
| 90 | tccagcgtgcgcata                       | sos |
| 91 | tctcccagcgtgcgcatt                    | o   |
| 92 | tccatgagcttcctgagtc                   | o   |
| 93 | gcatgtcgtttagct                       | sos |
| 94 | tcctgacgttcctgacgtt                   | s   |

|     |  |     |
|-----|--|-----|
| 95  | gcatgatgtttagct                                | o   |
| 96  | gcatttcgaggagct                                | o   |
| 97  | gcatgttagcttagct                               | o   |
| 98  | tccaggacgttccttagttct                          | o   |
| 99  | tccaggagcttccttagttct                          | o   |
| 100 | tccaggatgttccttagttct                          | o   |
| 101 | tccagtctaggccttagttct                          | o   |
| 102 | tccagttcgagccttagttct                          | o   |
| 103 | gcatggcggttagct                                | sos |
| 104 | gcatagcggttagct                                | sos |
| 105 | gcattgcgttagct                                 | sos |
| 106 | gcttgcgttgcgtt                                 | sos |
| 107 | tctcccagcggtgcgcataat                          | sos |
| 108 | tctcccagcggtgcgttatat                          | sos |
| 109 | tctccctgcgtgcgcataat                           | sos |
| 110 | tctgcgtgcgtgcgcataat                           | sos |
| 111 | tctcctagcgtgcgcataat                           | sos |
| 112 | tctcccagcggtgcgccttt                           | sos |
| 113 | gctandcghhage                                  | o   |
| 114 | tcctgacgttccc                                  | o   |
| 115 | ggaagacgtttaga                                 | o   |
| 116 | tcctgacgtttaga                                 | o   |
| 117 | tcagaccagctggtcgggtgttccctga                   | o   |
| 118 | tcaggaacacccgaccagctggtctga                    | o   |
| 119 | gctagtcgatagc                                  | o   |
| 120 | gctagtcgttagc                                  | o   |
| 121 | gcttgacgtctagc                                 | o   |
| 122 | gcttgacgtttagc                                 | o   |
| 123 | gcttgacgtcaagc                                 | o   |
| 124 | gctagacgtttagc                                 | o   |
| 125 | tccatgacattcctgatgct                           | o   |
| 126 | gctagacgtctagc                                 | o   |
| 127 | ggctatgtcggttcctagcc                           | o   |
| 128 | ggctatgtcgatcctagcc                            | o   |
| 129 | ctcatgggttctccaccaag                           | o   |
| 130 | cttgggtggagaaacccatgag                         | o   |
| 131 | tccatgacgttccatgttct                           | o   |
| 132 | ccgcttcctccagatgagctcatg                       | o   |
| 133 | catgagctcatctggaggaagcgg                       | o   |
| 134 | ccagatgagctcatgggttctcc                        | o   |
| 135 | ggagaaaacccatgagctcatctgg                      | o   |
| 136 | agcatcaggaacgacatgga                           | o   |
| 137 | tccatgacgttccatgttct                           | rna |
| 138 | gcgcgcgcgcgcgcgcgcgc                           | o   |
| 139 | ccggccggccggccggccgg                           | o   |
| 140 | ttccaatcagccccaccccgctctggccccacccctcacccctcca | o   |
| 141 | tggagggtgagggtggggccagagcgggtgggctgattgaa      | o   |
| 142 | tcaaattgtggatttccatgatct                       | o   |
| 143 | agactcatggaaaatccacatgttga                     | o   |
| 144 | tgccaagtgtgagtcactaataaaaga                    | o   |
| 145 | tctttatttagtgcactcagcaacttgca                  | o   |

|     |   |     |
|-----|---|-----|
| 146 | tgcaggaaagtccgggtttccccaaacccccc              | o   |
| 147 | gggggggttggggaaaacccgacttcctgca               | o   |
| 148 | ggggactttcccgctgggacttccagggggacttcc          | sos |
| 149 | tccatgacgttccctccatgacgttccctccatgacgttcc     | o   |
| 150 | gaggaacgtcatggagaggaacgtcatggagaggaacgtcatgga | o   |
| 151 | ataatagagcttcaagcaag                          | s   |
| 152 | tccatgacgttccgtacgtt                          | s   |
| 153 | tccatgacgttccgtacgtt                          | sos |
| 154 | tccaggacttccctcagggt                          | s   |
| 155 | tcttgcgtatctaaaggacgtcacattgcacaatcttaataaggt | o   |
| 156 | accttattaagattgtcaatgtacgtccttagcatcgcaaga    | o   |
| 157 | tcctgacgttccctggcggtccgtcgct                  | o   |
| 158 | tcctgtcgctccgtcgct                            | o   |
| 159 | tcctgacgttgaagt                               | o   |
| 160 | tcctgtcggtgaagt                               | o   |
| 161 | tcctggcggtgaagt                               | o   |
| 162 | tcctgccgttgaagt                               | o   |
| 163 | tccttacgttgaagt                               | o   |
| 164 | tcctaacgttgaagt                               | o   |
| 165 | tcctcacgttgaagt                               | o   |
| 166 | tcctgacgttgaagt                               | o   |
| 167 | tcctgacgttgaagt                               | o   |
| 168 | tcctgacgggtgaagt                              | o   |
| 169 | tcctgacgttgaagt                               | o   |
| 170 | tcctgacgttgaagt                               | o   |
| 171 | tcctgacgttgaagt                               | o   |
| 172 | tcctgagcttgaagt                               | o   |
| 173 | gggggacgttgggg                                | o   |
| 174 | tcctgacgttccctc                               | o   |
| 175 | tctcccaagcgagcgagcgccat                       | s   |
| 176 | tcctgacgttccctggcggtccctgtcgct                | o   |
| 177 | tcctgtcgcttctgtcgcttctgtcgct                  | o   |
| 178 | tcctggggggaaagt                               | o   |
| 179 | tcctgazgttgaagt                               | o   |
| 180 | tcztgacgttgaagt                               | o   |
| 181 | tccttaggttgaagt                               | o   |
| 182 | tccagacgttgaagt                               | o   |
| 183 | tcctgacggggaaagt                              | o   |
| 184 | tcctggcggttgaagt                              | o   |
| 185 | ggctccggggagggaaattttgttat                    | o   |
| 186 | atagacaaaaattccctccccggagcc                   | o   |
| 187 | tccatgagcttccgtttagtct                        | rna |
| 188 | tctgtcgctgtcccgcttctt                         | so  |
| 189 | tctgtcgctgtcccgcttctt                         | s20 |
| 190 | tctgagacattgcacaatcatctg                      | o   |
| 191 | cagattgtcaatgtctcg                            | o   |
| 192 | tccatgtcggttccgtatgcg                         | o   |
| 193 | gcgtatgtcggttccgtatgcg                        | o   |
| 194 | gcgtatgtcggttccgtatgcg                        | o   |
| 195 | tccatgtcggttccgtatgcg                         | o   |
| 196 | tccatgtcggttccgtatgcg                         | o   |

|     |                                     |          |
|-----|-------------------------------------|----------|
| 197 | tccatgtcgccccctgttagct              | o        |
| 198 | gcggcgccccccccccccccc               | o        |
| 199 | atcaggaacgtcatggaaagc               | o        |
| 200 | tccatgagccccctgagtc                 | p-ethoxy |
| 201 | tcaacgtt                            | p-ethoxy |
| 202 | tcaagctt                            | p-ethoxy |
| 203 | tcctgtcgccccctgtcgtt                | s        |
| 204 | tccatgtcgcccccccccc                 | s        |
| 205 | tcctgtcgcccccccccc                  | s        |
| 206 | tcctgtcgcccccccccc                  | s        |
| 207 | btccattccatgacgtccccatgtcgatgtttcca | os       |
| 208 | tcctgtcgcccccccccc                  | s        |
| 209 | tctgtcgctgtcccgcccttt               | s        |
| 210 | tctgtcgctgtcccgcccttt               | s        |
| 211 | tctgtcgctgtcccgcccttt               | s        |
| 212 | tcctgtcgccccccccccgttggAACGACAGG    | o        |
| 213 | tcctgtcgccccccccccgttggAACGACAGG    | o        |
| 214 | gggggtctgtcgcccccccccc              | sos      |
| 215 | gggggtctgtcgcccccccccc              | sos      |
| 216 | tccggccgttgaagt                     | o        |
| 217 | tccggacggtgaagt                     | o        |
| 218 | tccggccgttgaagt                     | o        |
| 219 | tccagacggtgaagt                     | o        |
| 220 | tccccacggtgaagt                     | o        |
| 221 | tccagagcttgaagt                     | o        |
| 222 | tccatgtzgtcccccccccc                | s        |
| 223 | tccatgacgtcccccccccc                | sos      |
| 224 | gggggttgcgtcccccccccc               | sos      |
| 225 | tccaggacttctctcaggcc                | s        |
| 226 | tttttttttttttttttttt                | s        |
| 227 | tccatgcgtcccccccccc                 | s        |
| 228 | tccatggcccccccccccc                 | s        |
| 229 | tccatgacgtcccccccccc                | s        |
| 230 | tccatgacgtcccccccccc                | s        |
| 231 | tccatgacgtcccccccccc                | s        |
| 232 | tccatgacggcccccccccc                | s        |
| 233 | tccatgcgtcccccccccc                 | s        |
| 234 | tccatgcgtcccccccccc                 | s        |
| 235 | btccattccatcttagccctgagtcgtttccat   | os       |
| 236 | tccatagcgcccccccccc                 | o        |
| 237 | tccatgtcgcccccccccc                 | o        |
| 238 | tccatagcgatcccccccccc               | o        |
| 239 | tccattgcgtcccccccccc                | o        |
| 240 | tccatagcgcccccccccc                 | o        |
| 241 | tccatgatcccccccccccc                |          |
| 242 | tccatgacgtcccccccccc                | s        |
| 243 | ggcgcccccccccccccccc                | o        |
| 244 | tccacacgtttcccgacgtt                | s        |
| 245 | tctgtcgcccccccccc                   | s        |
| 246 | tctgtcgcccccccccc                   | s        |
| 247 | tctgtcgcccccccccc                   | s        |

|     |  |     |
|-----|--|-----|
| 248 | gctgcgttgtcgttgtcgtt                     | s   |
| 249 | czggczggczggczccgg                       | o   |
| 250 | gccccggcgccgcgcgc                        | s   |
| 251 | agccccgigaacgattcac                      | o   |
| 252 | tgtcgtttgtcgttgtcgtt                     | s   |
| 253 | tgtcgttgtcgttgtcgttgtcgtt                | s   |
| 254 | tgtcgttgtcgttgtcgttgtcgtt                | s   |
| 255 | tctgtcgtcgtcgtt                          | s   |
| 256 | tgtcgttgtcgtt                            | s   |
| 257 | cccccccccccccccccccc                     | s   |
| 258 | tctagcgttttagcgttcc                      | sos |
| 259 | tgcattcccccaggccaccat                    | s   |
| 260 | tctgtcgtcgtcgtcgtcgtt                    | sos |
| 261 | tctgtcgttgtcgttgtcgtt                    | sos |
| 262 | tctgtcgtttgtcgtttgtcgtt                  | sos |
| 263 | tctgtcgttgtcgtttgtcgtt                   | sos |
| 264 | ggggaggggaggaacttcttaaaattcccccagaatgttt | o   |
| 265 | aaacattctggggaaatttaagaagttccctccccc     | o   |
| 266 | atgtttacttcttaaaattcccccagaatgttt        | o   |
| 267 | aaacattctggggaaatttaagaagtaaacat         | o   |
| 268 | atgtttacttagacaaaattcccccagaatgttt       | o   |
| 269 | aaacattctggggaaatttgcgttagtaaacat        | o   |
| 270 | aaaattgacgtttaaaaaa                      | sos |
| 271 | cccttgacgtttcccccc                       | sos |
| 272 | tttgcgttgtttgtcgtt                       |     |
| 273 | tctgtcgtttgtcgtttgtcgtt                  | sos |
| 274 | ctgcagccctggac                           | o   |
| 275 | accgcgttaattatagtaaaaccc                 | o   |
| 276 | ggtagccctgtggggacattgt                   | o   |
| 277 | agcaccaacgtgagagg                        | o   |
| 278 | tccatgccgttccctggcggt                    | o   |
| 279 | tccatgacggtcctgacgg                      | o   |
| 280 | tccatgccggtcctggcggt                     | o   |
| 281 | tccatgcgcgtcctgcgcgt                     | o   |
| 282 | ctggtctttctgggtttttctgg                  | s   |
| 283 | tcaagggtggggggaaacctt                    | sos |
| 284 | tccatgazgttccctagttt                     | o   |
| 285 | tccatgatgttccctagttt                     | o   |
| 286 | cccaagtcattccctttaacctgg                 | o   |
| 287 | ccaggtaagaggaaatgacttcggg                | o   |
| 288 | tccctggzggggaaagt                        | o   |
| 289 | gzggzggzggzgzzcccc                       | x   |
| 290 | tccatgtgttccctgatgt                      | o   |
| 291 | tccatgttccctgatgt                        |     |
| 292 | tccatgtcggtccctagttt                     |     |
| 293 | tccaagtagttccctagttt                     | o   |
| 294 | tccatgttagttccctagttt                    | o   |
| 295 | tcccgccgttcccgccgtt                      | s   |
| 296 | tccctggcggttccctggcggtt                  | s   |
| 297 | tccctggagggggaaagt                       | o   |
| 298 | tccctggggggggaaagt                       | o   |

|     |                                    |     |
|-----|------------------------------------|-----|
| 299 | tcctggtgggaagt                     | o   |
| 300 | tgcgttttgcgtttgcgtt                | o   |
| 301 | ctggtcctcggttttctgg                | o   |
| 302 | tccatgacgttctgacgtt                | o   |
| 303 | tccaggacttctcaggtt                 | sos |
| 304 | tzgtzgttttzgttttzgtt               | o   |
| 305 | btcgtcgccccgttttcgtttttt           | os  |
| 306 | gctatgacgttccaagggg                | s   |
| 307 | tcaacgtt                           | s   |
| 308 | tccaggacttccctcaggtt               | o   |
| 309 | ctctctgttaggcccgttgg               | s   |
| 310 | cttccgttgaccctggg                  | s   |
| 311 | gtccggggccaggccaaatc               | s   |
| 312 | gtgcgcgcgagcccaaatc                | s   |
| 313 | tccatgaigttcctgaigtt               | s   |
| 314 | aatagtcgcataaaaaaac                | o   |
| 315 | aatagtcgcatggcggggc                | o   |
| 316 | bttttccatgtcgccccgtttttt           | os  |
| 317 | tccgtcggttaagttttt                 | o   |
| 318 | gctagcttttagagctttagagtt           | o   |
| 319 | tgcgtctcccccccccccc                | o   |
| 320 | tcgacgttcccccccccccc               | o   |
| 321 | tgcgttcccccccccccc                 | o   |
| 322 | tgcgttcccccccccccc                 | o   |
| 323 | tcgcgttcccccccccccc                | o   |
| 324 | tgcgtgatcccccccccccc               | o   |
| 325 | tccgtacgttgaagt                    | s   |
| 326 | tccgtccgttgaagt                    | s   |
| 327 | tccgtacggtgaagt                    | s   |
| 328 | tccgtagcttgaagt                    | s   |
| 329 | tccgtggcgggaaagt                   | s   |
| 330 | aaaatctgtgtttttaaaaaa              | sos |
| 331 | gatccagtcacagtgcacctggcagaatctggat | o   |
| 332 | gatccagattctgccaggtaactgtactggat   | o   |
| 333 | gatccagtcacagtgactcagcagaatctggat  | o   |
| 334 | gatccagattctgtgagtcactgtactggat    | o   |
| 335 | tgcgttcccccccccccc                 | o   |
| 336 | tzgtqgttcccccccccccc               | o   |
| 337 | tzgtcggtcccccccccccc               | o   |
| 338 | tcgtzgttcccccccccccc               | o   |
| 339 | tgcgtcggtcccccccccccc              | o   |
| 340 | tgcgtcggtcccccccccccc              | o   |
| 341 | tcggcggttcccccccccccc              | o   |
| 342 | ggcttttcccccccccccc                | o   |
| 343 | tgcgttttgcgttttgcgtt               | s   |
| 344 | tgcgttttgcgttttgcgtt               | s   |
| 345 | ccgtcggtcccccccccccc               | o   |
| 346 | gcgtcggtcccccccccccc               | o   |
| 347 | tgcgtattcccccccccccc               | o   |
| 348 | acgtcggtcccccccccccc               | o   |
| 349 | ctgtcggtcccccccccccc               | o   |

|     |  |     |
|-----|--|-----|
| 350 | btttttcgtcgttcccccccccccc              | os  |
| 351 | tgcgtgtcccccccccccb                    | o   |
| 352 | tgcgtgtttgtcggttgcgttb                 | o   |
| 353 | tccagttccctcctcagtc                    | o   |
| 354 | tzgtcggttgtcggttgtcgtt                 | o   |
| 355 | tcctggagggaagt                         | s   |
| 356 | tcttggaaaaggaaagt                      | s   |
| 357 | tgcgtgtcccccccccc                      | s   |
| 358 | tzgtzggttgtzgttgtzgtt                  | s   |
| 359 | ggggtaagcttgagggggg                    | sos |
| 360 | tgcgtgtcccccccccc                      | s   |
| 361 | tgcgtcgtcgtcgtt                        | s2  |
| 362 | tgcgtcgtcgtcgtt                        | s20 |
| 363 | tgcgtcgtcgtcgtt                        | os2 |
| 364 | tcaacgttga                             | s   |
| 365 | tcaacgtt                               | s   |
| 366 | atagtttccatttttttac                    |     |
| 367 | aatagtcgccatcgcgac                     | o   |
| 368 | aatagtcgccatcccggac                    | o   |
| 369 | aatagtcgccatccccccc                    | o   |
| 370 | tgcgtgtttgtgtttgtgtt                   | o   |
| 371 | ctgtgtttctgtgtttttctgt                 | s   |
| 372 | ctaattttctaattttttctaa                 | s   |
| 373 | tgcgtgtgtgtcggtgtcgtt                  | s   |
| 374 | tgcgtgtgtgtcggtgtgtt                   | s   |
| 375 | accatggacgagctgtttccctc                |     |
| 376 | tgcgtgttgcgtgcgtt                      | s   |
| 377 | ctgttaagttagactttggagag                |     |
| 378 | gagaacgctggacccctcc                    |     |
| 379 | cggcgactcagtctatcg                     |     |
| 380 | gttctcagataaaagcgaaaccagcaacagacacagaa |     |
| 381 | ttctgtgtctgtgtgtgtgtttccgtttatctgagaac |     |
| 382 | cagacacagaagccccgatagacg               |     |
| 383 | agacagacacgaaacgaccg                   |     |
| 384 | gtctgtcccatgtatctcgaa                  |     |
| 385 | gctggccagcttacccccc                    |     |
| 386 | ggggcctctataacaacctgg                  |     |
| 387 | ggggccctgagactgcc                      |     |
| 388 | gagaacgctggacccctccat                  |     |
| 389 | tccatgtcggtcctgatgct                   |     |
| 390 | ctcttgcgacccgtggaaaggta                |     |
| 391 | aggtacagccaggactacga                   |     |
| 392 | accatggacgacccgtttccctc                |     |
| 393 | accatggattacccctttccctt                |     |
| 394 | atggaaaggccagcggttc                    | o   |
| 395 | acgtatcaggaccgacatgg                   | o   |
| 396 | ccttccaagctcaacttacag                  |     |
| 397 | tccctgagactgccccacctt                  |     |
| 398 | gccacccaaaacttgtccatg                  |     |
| 399 | gtccatggcggtcgccggatga                 |     |
| 400 | cctctataacaacctggac                    |     |



|     |                               |
|-----|-------------------------------|
| 452 | gtagccttccta                  |
| 453 | cggtagccttccta                |
| 454 | cacggtagccttccta              |
| 455 | agcacggtagccttccta            |
| 456 | gaacgctggacccat               |
| 457 | gacccttccat                   |
| 458 | tggacccat                     |
| 459 | gctggacccat                   |
| 460 | acgctggacccat                 |
| 461 | taagctctgtcaacgccagg          |
| 462 | gagaacgctggacccatgt           |
| 463 | tccatgtcggtccatgt             |
| 464 | ttcatgccttgcaaaatggcg         |
| 465 | tgcctgtgtgcctgtact            |
| 466 | agcatcaggaccgacatgg           |
| 467 | gaccctccatgtcggtccat          |
| 468 | acaaccacgagaacgggaa           |
| 469 | gaacccatgtgttccg              |
| 470 | caatcaatctgaggagaccc          |
| 471 | tcagctctgttacttttca           |
| 472 | tgttacggctgtcccatt            |
| 473 | gtctatcgaggactggcg            |
| 474 | catttacggcgccggcg             |
| 475 | gaggggaccatttacggc            |
| 476 | tgtccagccgaggggaccat          |
| 477 | cgggcttacggcgatgt             |
| 478 | tggacccatgtcggtcc             |
| 479 | tgtccatgttttagaagc            |
| 480 | gtggttacggctgtccccat          |
| 481 | cctccaaatgaaagacccc           |
| 482 | ttgtactctccatgtgtt            |
| 483 | ttccatgtgtccggctgg            |
| 484 | gaccctctatgtcggtcc            |
| 485 | gagaccgctcgaccctcgat          |
| 486 | ttggcccatattttagaaac          |
| 487 | ttgaaaactgaggtggac            |
| 488 | ctatcgaggactggcgcc            |
| 489 | cttggaggccctccggcg            |
| 490 | gctgaacccatgtgtt              |
| 491 | tagaaacagcatttttttagggcagcaca |
| 492 | agatggttctcagataaagcgaa       |
| 493 | ttccgcattatctgagaaccatct      |
| 494 | gtcccaggttgtatacgaggtgc       |
| 495 | gcgcgcgtcccgatagac            |
| 496 | atcggaggactggcgccg            |
| 497 | ggctgtccatattttag             |
| 498 | ttttcaacgttgagggggg           |
| 499 | ttttcaagcggtgatTTTT           |
| 500 | ggggcaacgttgatTTTT            |
| 501 | ggggTTTcaacgtttgagggggg       |
| 502 | ggtaacggctgtccat              |

sos

sos

sos

sos

|     |                                     |     |
|-----|-------------------------------------|-----|
| 503 | ctgtcccatatTTTtagaca                |     |
| 504 | accatcctgaggccattcgg                |     |
| 505 | cgtctatcgggcttctgtgtctg             |     |
| 506 | ggccatcccacattgaaagt                |     |
| 507 | ccaaatatcggtggtcaagcac              |     |
| 508 | gtgcttgaccaccgatatttgg              |     |
| 509 | gtgctgatcaccgatatcctgttcgg          |     |
| 510 | ggccaacttcaatgtggatggcctc           |     |
| 511 | tccgcgcgaatggcctcaggatggtaC         |     |
| 512 | tatagtcctcgagactgccccaccccttcacaacc |     |
| 513 | gcagcctctatacaacctggacggga          |     |
| 514 | ctatcgaggactggcgccg                 |     |
| 515 | tatcgaggactggcgccg                  |     |
| 516 | gatcgaggactggcgccg                  |     |
| 517 | ccgaacaggatatcggtgatcagcac          |     |
| 518 | tttgggtcaacgttgggggggg              |     |
| 519 | ggggtaacgttgggggggg                 | sos |
| 520 | cgcgcgcgcgcgcgcgcgc                 | s   |
| 521 | ggggcatgacgttcccccccc               | ss  |
| 522 | ggggcatgacgttcccccccc               | s   |
| 523 | ggggcatgacgttcccccccc               | s   |
| 524 | ggggcatgacgttcccccccc               | sos |
| 525 | aaaacatgacgttcccccccc               | sos |
| 526 | aaaacatgacgttcccccccc               | sos |
| 527 | ggggcatgacgttcccccccc               | sos |
| 528 | accatggacgatctgtttccccctc           | s   |
| 529 | gccatggacgaaactgttccccctc           | s   |
| 530 | cccccccccccccccccccc                | sos |
| 531 | gggggggggggggggggggggggg            | sos |
| 532 | gtgtaaaatgaaatcgccg                 | sos |
| 533 | ttcggcgactcctccatt                  | sos |
| 534 | tatgcgcgcggacttat                   | sos |
| 535 | gggttaatcgatcagggggg                | sos |
| 536 | tttggacgttccatgttc                  | sos |
| 537 | gatcgctgatctaattgtcg                | sos |
| 538 | gtcggtctgtatgtttcc                  | sos |
| 539 | tctcgatcgatcgatcgatcg               | sos |
| 540 | ctggacccatgtcg                      | sos |
| 541 | gctcgatcgatcgatcg                   | sos |
| 542 | ctggacccatgtcg                      | sos |
| 543 | cactgtccatgtcg                      | sos |
| 544 | cgtggacccatgtcg                     | sos |
| 545 | gctgatcgatcgatcg                    | sos |
| 546 | aacgtggacccatgtcg                   | sos |
| 547 | tgcgttgcgttgcgttgcgtt               | sos |
| 548 | cctccatgtcgatcgatcgat               | sos |
| 549 | tactttcgatccatgtcg                  | sos |
| 550 | ttccatgtcgatcgatcgat                | sos |
| 551 | ctgattgtcgatcgatcgat                | sos |
| 552 | ggcgatcgatcgatcgatcgat              | o   |
| 553 | cctacgttgcgttgcgttgcgtt             | o   |

|     |  |   |
|-----|--|---|
| 554 | ggggtaatcgatgaggggg                            | o |
| 555 | ttcggggggactccatt                              | o |
| 556 | tttttttttttttttttt                             | o |
| 557 | gggggtttttttttttttt                            | o |
| 558 | ttttgggggggggggtttt                            | o |
| 559 | gggggggggggggggggt                             | o |
| 560 | aaaaaaaaaaaaaaaaaaa                            | o |
| 561 | cccccaaaaaaaaaaaaaaa                           | o |
| 562 | aaaaacccccccccccaaaa                           | o |
| 563 | tttgaattcaggacttgtgaggttag                     | o |
| 564 | tttgaatcttcagcgctccaggcgc                      | o |
| 565 | aattctctatcggggttctgtctgttgttttat              | o |
| 566 | ctagataaaagcggAACAGCAACAGACACAGAAAGCCCCGATAGAG | o |
| 567 | ttttctagagagggtgcacaatgtctgg                   | o |
| 568 | tttgaattccgtgtacagaagcgagaagc                  | o |
| 569 | tttgcggccgctagacttaacctgagagata                | o |
| 570 | tttggcccacgagagacagagacacttc                   | o |
| 571 | tttggcccgcttctcgcttctgtacacg                   | o |
| 572 | gagaacgtggacccat                               | s |
| 573 | tccatgtcggtcctgtatgt                           | s |
| 574 | ctgtcg   | s |
| 575 | tcgtga   | s |
| 576 | cgtcga   | s |
| 577 | agtgct   | s |
| 578 | ctgtcg   | o |
| 579 | agtgct   | o |
| 580 | cgtcga   | o |
| 581 | tcgtga   | o |
| 582 | gagaacgtccagcttcgat                            | o |
| 583 | gctagacgttaagcggtga                            | o |
| 584 | gagaacgctcgacccat                              | o |
| 585 | gagaacgctggacccat                              | o |
| 586 | gctagagggttagcggtga                            | o |
| 587 | gagaacgctggacttccat                            | o |
| 588 | tcacgctaacgtctagc                              | o |
| 589 | bgctagacgttagcggtga                            | o |
| 590 | atggaaagggtcgagcggtctc                         | o |
| 591 | gagaacgctggacccat                              | o |
| 592 | gagaacgatggacccat                              | o |
| 593 | gagaacgctggatccat                              | o |
| 594 | gagaacgctccagcactgat                           | o |
| 595 | tccatgtcggtcctgtatgt                           | o |
| 596 | atgtcctcggtcctgtatgt                           | o |
| 597 | gagaacgctccacccat                              | o |
| 598 | gagaacgctggacccat                              | o |
| 599 | batggaaagggtccagcggtctc                        | o |
| 600 | tcctgt   | o |
| 601 | tcaacgtt                                       | o |
| 602 | aacgtt   | o |
| 603 | aacgttga                                       | o |
| 604 | tcacgctaacccttagc                              | o |

|     |                         |   |
|-----|-------------------------|---|
| 605 | gagaacgctggacccat       | o |
| 606 | gctggacccat             | o |
| 607 | gagaacgctggacccatccat   | o |
| 608 | gagaacgctggacgctcatccat | o |
| 609 | aacgttggggcat           | o |
| 610 | atgcaccaaacgtt          | o |
| 611 | tcaacgttga              | o |
| 612 | gctggacccat             | o |
| 613 | caacgtt                 | o |
| 614 | acaacgttga              | o |
| 615 | tcacgt                  | o |
| 616 | tcaagctt                | o |
| 617 | tctgtca                 | o |
| 618 | aggatatac               | o |
| 619 | tagacgtc                | o |
| 620 | gacgtcat                | o |
| 621 | ccatcgat                | o |
| 622 | atcgatgt                | o |
| 623 | atgcatgt                | o |
| 624 | ccatcgat                | o |
| 625 | agcgctga                | o |
| 626 | tcagcgct                | o |
| 627 | ccttcgat                | o |
| 628 | gtgccggggctccgggc       | s |
| 629 | gctgtggggccgcctcg       | s |
| 630 | btcAACGTT               | o |
| 631 | ftcaacgtt               | o |
| 632 | faacgttga               | o |
| 633 | tcaacgt                 | s |
| 634 | aacgttg                 | s |
| 635 | cgacga                  | o |
| 636 | tcaacgtt                | o |
| 637 | tcgga                   | o |
| 638 | agaacgtt                | o |
| 639 | tcatcgat                | o |
| 640 | taaacgtt                | s |
| 641 | ccaacgtt                | s |
| 642 | gctcgaa                 | s |
| 643 | cgacgt                  | s |
| 644 | cgtcgat                 | s |
| 645 | acgtgt                  | s |
| 646 | cgttcg                  | s |
| 647 | gagcaagctggacccat       | s |
| 648 | cgcgtt                  | s |
| 649 | cgtacg                  | s |
| 650 | tcaccggat               | s |
| 651 | caagagatgctaacaatgca    | s |
| 652 | accatcaatagctctgtgc     | s |
| 653 | ccatcgat                | o |
| 654 | tcgacgtc                | o |
| 655 | ctagcgct                | o |

|     |                           |   |
|-----|---------------------------|---|
| 656 | taagcgct                  | o |
| 657 | tccgcgaattcgcg            | o |
| 658 | atggaaagggtccagcgttct     | o |
| 659 | actggacgttagcgtga         | o |
| 660 | cgcctggggctggtctgg        | o |
| 661 | gtgtcggggtctccgggc        | o |
| 662 | gtccgggggtctccgggc        | o |
| 663 | cgcgcgtcgccgcggttgg       | o |
| 664 | gaagttcacgttgaggggcat     | s |
| 665 | atctggtgaggggcaagctatg    | s |
| 666 | gttcaaaccgagaacatcat      | o |
| 667 | gcaacgtt                  | o |
| 668 | gtaacgtt                  | o |
| 669 | cgaacgtt                  | o |
| 670 | gaaacgtt                  | o |
| 671 | caaacgtt                  | o |
| 672 | ctaacgtt                  | o |
| 673 | ggaacgtt                  | o |
| 674 | tgaacgtt                  | o |
| 675 | acaacgtt                  | o |
| 676 | ttaacgtt                  | o |
| 677 | aaaacgtt                  | o |
| 678 | ataacgtt                  | o |
| 679 | aacgttct                  | o |
| 680 | tccgatcg                  | o |
| 681 | tccgtacg                  | o |
| 682 | gctagacgtacgtga           | o |
| 683 | gagaacgtggacacctatcatccat | o |
| 684 | gagaacgttagaccttctat      | o |
| 685 | actagacgttagtgtga         | o |
| 686 | cacaccttggtaatgtcacgt     | o |
| 687 | tctccatctatggtttatcg      | o |
| 688 | cgtggacacctccat           | o |
| 689 | caccaccttggtaatgtcacgt    | o |
| 690 | gctagacgttagtggaa         | o |
| 691 | agtgcgatttcgagatcg        | o |
| 692 | tttcgttttgggtttgtggtt     |   |
| 693 | tttcgtttgtcggtttgtcggt    |   |
| 694 | ttttgtttgtggttttgtggtt    | s |
| 695 | accgcattggattctaggcca     | o |
| 696 | gctagacgttagcgt           | o |
| 697 | aacgctggacacctccat        | o |
| 698 | tcaazgtt                  | o |
| 699 | ccttcgat                  | o |
| 700 | actagacgttagtgtga         | s |
| 701 | gctagagggttagcgtga        | s |
| 702 | atggactctccagcgttctc      | o |
| 703 | atcgactctcgagcgttctc      | o |
| 704 | gctagacgttagc             | o |
| 705 | gctagacgt                 | o |
| 706 | agtgcgattcgagatcg         | o |

|     |   |     |
|-----|---|-----|
| 707 | tca g z g c t                               | o   |
| 708 | c t g a t t g c t c t c g t g a             | o   |
| 709 | t z a a c g t t                             | o   |
| 710 | g a g a a z g c t g g a c c t t c c a t     | o   |
| 711 | g c t a g a c g t t a g g c t g a           | o   |
| 712 | g c t a c t t a g c g t g a                 | o   |
| 713 | g c t a c c t t a g c g t g a               | o   |
| 714 | a t c g a c t t c g a g c g t t c t c       | o   |
| 715 | a t g c a c t t c g a c g c g t t c t c     | o   |
| 716 | a g t g a c t t c c a g c g t t c t c       | o   |
| 717 | g c c a g a t g t t a g c t g g a           | o   |
| 718 | a t c g a c t c g a g c g t t c t c         | o   |
| 719 | a t c g a t c g a g c g t t c t c           | o   |
| 720 | b g a g a a c g c t c g a c c t t c g a t   | sos |
| 721 | g c t a g a c g t t a g c t g g a           | sos |
| 722 | a t c g a c t t c t c g a g c g t t c t c   | o   |
| 723 | t a g a c g t t a g c g t g a               | o   |
| 724 | c g a c t t c t c g a g c g t t c t c       | o   |
| 725 | g g g g t c g a c c t t g g a g g g g g g g | sos |
| 726 | g c t a a c g t t a g c g t g a             | o   |
| 727 | c g t c g t c g t                           | o   |
| 728 | g a g a a c g c t g g a c z t t c c a t     | o   |
| 729 | a t c g a c c t a c g t g c g t t z t c     | o   |
| 730 | a t z g a c c t a c g t g c g t t c t c     | o   |
| 731 | g c t a g a z g t t a g c g t               | o   |
| 732 | a t c g a c t t c g a g z g t t c t c       | o   |
| 733 | g g g g t a a t g c a t c a g g g g g g     | sos |
| 734 | g g c t g t a t t c c t g a c t g c c c     | s   |
| 735 | c c a t g c t a a c c t c t a g c           | o   |
| 736 | g c t a g a t g t t a g c g t g a           | o   |
| 737 | c g t a c c t t a c g g t g a               | o   |
| 738 | t c c a t g c t g g t c c t g a t g c t     | o   |
| 739 | a t c g a c t t c t c g a g c g t t c t c   | o   |
| 740 | g c t a g a g c t t a g c g t g a           | o   |
| 741 | a t c g a c t t c g a g t g t t c t c       | o   |
| 742 | a a c g t c g a c c t t c g a t             | o   |
| 743 | c t c a a c g t g g a c c t t c c a t       | o   |
| 744 | a t c g a c c t a c g t g c g t t c t c     | o   |
| 745 | g a g a a t g c t g g a c c t t c c a t     | o   |
| 746 | t c a c g c t a a c c t c t g a c           | o   |
| 747 | b g a g a a c g t c c a g c a c t g a t     | o   |
| 748 | b g a g c a a g c t g g a c c t t c c a t   | o   |
| 749 | c g c t a g a g g t t a g c g t g a         | o   |
| 750 | g c t a g a t g t t a a c g t               | o   |
| 751 | a t g g a a g g t c c a c g t t c t c       | o   |
| 752 | g c t a g a t g t t a g c g t               | o   |
| 753 | g c t a g a c g t t a g t g t               | o   |
| 754 | t c c a t g a c g g t c c t g a t g c t     | o   |
| 755 | t c c a t g g c g g t c c t g a t g c t     | o   |
| 756 | g c t a g a c g a t a g c g t               | o   |
| 757 | g c t a g t c g a t a g c g t               | o   |

|     |                             |     |
|-----|-----------------------------|-----|
| 758 | tccatgacgttccctgatgct       | o   |
| 759 | tccatgtcggtccctgatgct       | o   |
| 760 | gctagacgttagzgt             | o   |
| 761 | gctaggcgtagcgt              | o   |
| 762 | tccatgtzggccctgatgct        | o   |
| 763 | tccatgtcggtzctgatgct        | o   |
| 764 | atzgactctzgagzgttctc        | o   |
| 765 | atggaaggccactgtttctc        | o   |
| 766 | gcatgacgttgagct             | o   |
| 767 | ggggtaacgttgagggggg         | s   |
| 768 | ggggtaagtctgagggggg         | sos |
| 769 | cgcgcgcgcgcgcgcgcgc         | o   |
| 770 | cccccccccccccccccccccccc    | s   |
| 771 | cccccccccccccccccccccccc    | s   |
| 772 | tccatgtcgctccgtatct         | o   |
| 773 | gctaaacgttagcgt             | o   |
| 774 | tccatgtcgatccgtatgct        | o   |
| 775 | tccatgccggccctgatgct        | o   |
| 776 | aaaatcaacgttaaaaaaaaa       | sos |
| 777 | tccataaacgttccgtatgct       | o   |
| 778 | tggaggtcccaccgagatcgag      | o   |
| 779 | cgtcgtcgtcgtcgtcgt          | s   |
| 780 | ctgctgtcgtcgtcgtcgt         | s   |
| 781 | gagaacgctccgacccgtat        | s   |
| 782 | gctagatgttagcgt             | s   |
| 783 | gcatgacgttgagct             | s   |
| 784 | tcaatgtcgaf                 | o   |
| 785 | tcaacgttgaf                 | o   |
| 786 | tcaacgttgab                 | o   |
| 787 | gcaatattgcb                 | o   |
| 788 | gcaatattgcf                 | o   |
| 789 | agttgcaact                  | o   |
| 790 | tcttcgaa                    | o   |
| 791 | tcaacgtc                    | o   |
| 792 | ccatgtcggtccctgatgct        | o   |
| 793 | gttttatataatttggg           | o   |
| 794 | tttttgttgcgtttgtcggt        | o   |
| 795 | ttgggggggggtt               | s   |
| 796 | ggggttgggggtt               | s   |
| 797 | ggtgtgttaggtttgg            | o   |
| 798 | bgagaazgctcgacccgtat        | o   |
| 799 | tcaacgttaacgttaacgtt        | o   |
| 800 | bgagcaagztggacccat          | o   |
| 801 | bgagaazgtccagactgat         | o   |
| 802 | tcaazgttgax                 | o   |
| 803 | gzaatattgcx                 | o   |
| 804 | tgcgtttgtcgtttgcgtt         | o   |
| 805 | ctgcgttagcaatttaactgtg      | o   |
| 806 | tccatgacgttccctgatgct       | s   |
| 807 | tgcgtccgtgcacccgtacacagctct | s   |
| 808 | tgcgtccgtacacagctct         | s   |

|     |                                  |     |
|-----|----------------------------------|-----|
| 809 | tgcatcagctct                     | s   |
| 810 | tgcgtct                          | s   |
| 811 | cccccccccccccccccc               | s   |
| 812 | cccccccccccc                     | s   |
| 813 | cccccccc                         | s   |
| 814 | tgcacgtct                        | sos |
| 815 | tgcacgttacacagctct               | o   |
| 816 | gagcaagctggaccattccat            | s   |
| 817 | tcaacgttaacgttaacgttaacgttaacgtt | s   |
| 818 | gagaacgctcgacccatcgat            | s   |
| 819 | gtccccatccccagaggaggaaat         | o   |
| 820 | ctagcggctgacgtcatcaagctag        | o   |
| 821 | ctagtttatgacgtcagccgttag         | o   |
| 822 | cggctgacgtcatcaa                 | s   |
| 823 | ctgacgtg                         | o   |
| 824 | ctgacgtcat                       | o   |
| 825 | attcgatccccccccccccgag           | o   |
| 826 | ctcgccccccccgatcaat              | o   |
| 827 | gactgacgtcagcgt                  | o   |
| 828 | ctagcggctgacgtcataaagctagc       | s   |
| 829 | ctagctttatgacgtcagccgttagc       | s   |
| 830 | ctagcggctgagctcataaagctagc       | s   |
| 831 | ctagtggctgacgtcatcaagctag        | s   |
| 832 | tccaccacgtggctatgct              | s   |
| 833 | ggaaatgaaagattttattataag         | o   |
| 834 | tctaaaaccatctattcttaaccct        | o   |
| 835 | agctcaacgtcatgc                  | o   |
| 836 | ttaacgggtggtagcggtattggtc        | o   |
| 837 | ttaagaccaataccgctaccaccg         | o   |
| 838 | gatctagtatgagtcagccggatc         | o   |
| 839 | gatccggctgactcatactagatc         | o   |
| 840 | tccaagacgttccctgatgct            | o   |
| 841 | tccatgacgtccctgatgct             | o   |
| 842 | tccaccacgtggctatgct              | o   |
| 843 | ccacgtggacaccttagc               | o   |
| 844 | tcaagaccacgtggtcgggtgttccctga    | o   |
| 845 | tcaagaaacaccgaccacgtggctgta      | o   |
| 846 | cattttccacgatttccca              | o   |
| 847 | tccctctctgcaagagact              | o   |
| 848 | tgtatctctgtaaaggact              | o   |
| 849 | ataaaacgtaaaactagcagcagtttc      | o   |
| 850 | gaaactgtgtctatgtttcgctttat       | o   |
| 851 | tgcggaaaagggaaaatttggcttccatcag  | o   |
| 852 | ctgtatgaaacaaaatttccctttggca     | o   |
| 853 | ttagggtttaggttagggtt             | ss  |
| 854 | tccatgacgttccctgatgct            | ss  |
| 855 | aaaacatgacgttcaaaaaa             | ss  |
| 856 | aaaacatgacgttccgggggg            | ss  |
| 857 | ggggcatgacgttccgggggg            | sos |
| 858 | ctaggctgacgtcatcaagctag          | o   |
| 859 | tctgacgtcatctgacgttggctgacgtct   | o   |



|     |                             |     |
|-----|-----------------------------|-----|
| 911 | ttttttttttttttttttttttttttt | s   |
| 912 | ttttttttttttttttttt         | s   |
| 913 | ttttttttttttttttt           | s   |
| 914 | gctagaggggagggt             |     |
| 915 | gctagatgttagggg             |     |
| 916 | gcatgagggggagct             |     |
| 917 | atggaaggccagggggctc         |     |
| 918 | atggactctggagggggctc        |     |
| 919 | atggaaggccaaggggctc         |     |
| 920 | gagaaggggggaccttggat        |     |
| 921 | gagaaggggggacccatccat       |     |
| 922 | gagaagggggcagcactgat        |     |
| 923 | tccatgtggggcctgatgct        |     |
| 924 | tccatgaggggcctgatgct        |     |
| 925 | tccatgtggggcctgctgat        |     |
| 926 | atggactctccgggggttc         |     |
| 927 | atggaaggccgggggttc          |     |
| 928 | atggactctggagggggttc        |     |
| 929 | atggaggctccatgggctc         |     |
| 930 | atggactctgggggttc           |     |
| 931 | tccatgtgggtggggatgct        |     |
| 932 | tccatgcgggtggggatgct        |     |
| 933 | tccatggggtcctgatgct         |     |
| 934 | tccatgggtccctgatgct         |     |
| 935 | tccatgggtgcctgatgct         |     |
| 936 | tccatgggttcctgatgct         |     |
| 937 | tccatcgggggcctgatgct        |     |
| 938 | gctagaggagtg                |     |
| 939 | ttttttttttttttt             | s   |
| 940 | gggggtcaacgttgggggggg       | s   |
| 941 | ggggagttcggtgggggggg        | s   |
| 942 | tctgtcgcccccccccccc         | s   |
| 943 | ttgggggggtttttttttttttt     | s   |
| 944 | tttaaatttaaaattttaaaata     | s   |
| 945 | ttggtttttttggtttttttgg      | s   |
| 946 | ttccctttccctttccctc         | s   |
| 947 | ggggtcatcgatgaggggg s       | sos |
| 948 | tccatgacgttcctgacgtt        |     |
| 949 | tccatgacgttcctgacgtt        |     |
| 950 | tccatgacgttcctgacgtt        |     |
| 951 | tccatgacgttcctgacgtt        |     |
| 952 | tccatgacgttcctgacgtt        |     |
| 953 | tccatgacgttcctgacgtt        |     |
| 954 | tccatgacgttcctgacgtt        |     |
| 955 | tccatgacgttcctgacgtt        |     |
| 956 | tccatgacgttcctgacgtt        |     |
| 957 | tccatgacgttcctgacgtt        |     |
| 958 | tccatgacgttcctgacgtt        |     |
| 959 | gggggacatcgccgggg           | sos |
| 960 | gggggtcgatcgacgggggg        | sos |
| 961 | ttttttttttttttttttt         | po  |

|      |                             |     |
|------|-----------------------------|-----|
| 962  | aaaaaaaaaaaaaaaaaaaaaaa     | po  |
| 963  | ccccccccccccccccccccccc     | po  |
| 964  | tcgtcgccccccccccccccccc     |     |
| 965  | tcgtcgccccccccccccccccc     |     |
| 966  | tcgtcgccccccccccccccccc     |     |
| 967  | tcgtcgccccccccccccccccc     |     |
| 968  | ggggcaacgttgggggggg         |     |
| 969  | ggggcaacgttgggggggg         |     |
| 970  | ggggcaacgttgggggggg         |     |
| 971  | tgcgttttttttttttttttt       |     |
| 972  | ggggacgtcgacgtgggggg        | sos |
| 973  | ggggtcgtcgacgagggggg        | sos |
| 974  | ggggtcgtacgtcgagggggg       | sos |
| 975  | ggggaccggtaaccgggggg        | sos |
| 976  | gggtcgtcgacgtcgaggggg       | sos |
| 977  | ggggtcgtacgtcgaggggg        | sos |
| 978  | ggggAACGTTAACCGTGGGGG       | sos |
| 979  | ggggtcaccgggtggggggg        | sos |
| 980  | ggggtcgttcaacgagggggg       | sos |
| 981  | ggggacgttcaacgtgggggg       | sos |
| 982  | tcaacttga                   | s   |
| 983  | tcaagcttga                  | s   |
| 984  | tcacgatcgtga                | s   |
| 985  | tcagcatgctga                | s   |
| 986  | gggggagcatgtcgaggggg        | sos |
| 987  | gggggggggggggggggggggg      | sos |
| 988  | gggggacgatatcgccgggggg      | sos |
| 989  | gggggacgacgtcgccgggggg      | sos |
| 990  | gggggacgacgtcgccgggggg      | sos |
| 991  | gggggacgtacgtcgaggggg       | sos |
| 992  | tcaacgtt                    |     |
| 993  | tccataccggctgtatgt          |     |
| 994  | tccataccggcttaccgg          | s   |
| 995  | gggggacgatcggtgggggg        | sos |
| 996  | ggggaaacgatcgccgggggg       | sos |
| 997  | ggg ggg acg atc gtc ggg ggg | sos |
| 998  | ggg gga cga tcg tcg ggg ggg | sos |
| 999  | aaa gac gtt aaa             | po  |
| 1000 | aaagagcttaaa                | po  |
| 1001 | aaagazgttaaa                | po  |
| 1002 | aaattcggaaaa                | po  |
| 1003 | gggggtcatcgatgagggggg       | sos |
| 1004 | gggggtcaacgttgggggggg       | sos |
| 1005 | atgtagcttataacaaggc         | po  |
| 1006 | ggatccctttagttacttct        | po  |
| 1007 | ccattccacccatgttacc         | po  |
| 1008 | tatgttattatcatgttagata      | po  |
| 1009 | agcctacgttattcacccctcc      | po  |
| 1010 | ttccctgcaactactattgtt       | po  |
| 1011 | atagaaggccctacaccagt        | po  |

|      |                                    |     |
|------|------------------------------------|-----|
| 1012 | ttacaccggctatggaggt                | po  |
| 1013 | ctaaccagatcaagtctagg               | po  |
| 1014 | cctagacttgatctggtag                | po  |
| 1015 | tataaggcctcgacatg                  | po  |
| 1016 | catgtcgacgaggctata                 | po  |
| 1017 | tgggtggggggagtaagctc               | po  |
| 1018 | gagctactccccaccacca                | po  |
| 1019 | gccttcgatcttgcgttggaa              | po  |
| 1020 | tggacttctcttgcgttct                | po  |
| 1021 | atgctgttagccagcgataa               | po  |
| 1022 | accqaatcagcggaaagtga               | po  |
| 1023 | tccatgacgttctgacgtt                |     |
| 1024 | ggagaaaaccatgagctcatctgg           |     |
| 1025 | accacagaccaggcaggcaga              |     |
| 1026 | gagcgtgaactgcgcgaaga               |     |
| 1027 | tcgggtacccttgcagcggtt              |     |
| 1028 | ctggagccctagccaaggat               |     |
| 1029 | gcgactccatcaccagcgat               |     |
| 1030 | cctgaagtaagaaccagatgt              |     |
| 1031 | ctgtgttatctgacatacacc              |     |
| 1032 | aattagccttaggtattgggg              |     |
| 1033 | acatctggttcttacttcagg              |     |
| 1034 | ataagtcatatttggaaactac             |     |
| 1035 | cccaatcacctaaggctaatt              |     |
| 1036 | ggggtcgtcgacgagggggg               | sos |
| 1037 | ggggtcgttcgaacgagggggg             | sos |
| 1038 | ggggacgttcgaacgtgggggg             | sos |
| 1039 | tcctggcgqggaaagt                   | s   |
| 1040 | ggggAACGACGTCGTGGGGGG              | sos |
| 1041 | ggggAACGTACGTCGGGGGG               | sos |
| 1042 | ggggAACGTACGTCGTGGGGGG             | sos |
| 1043 | gggtcaccgggtgagggggg               | sos |
| 1044 | gggtcgtacgtcgagggggg               | sos |
| 1045 | ggggaccgggtaccgggtgggggg           | sos |
| 1046 | gggtcgtacgtcgagggggg               | sos |
| 1047 | gggtcgtacgtcgagggggg               | sos |
| 1048 | ggggAACGTTAACGTTGGGGGG             | sos |
| 1049 | ggggacgtcgacgtgggggg               | sos |
| 1050 | gcactcttcgaagctacagccggcagcctctgat |     |
| 1051 | cggctctccatgaggctttgtaatcttgg      |     |
| 1052 | cggctctccatgaaagtcttggacgtgtgagc   |     |
| 1053 | tcctgcaggtaagt                     | s   |
| 1054 | gggggtcgttcgtgggggggg              | sos |
| 1055 | gggggatgattgtgggggggg              | sos |
| 1056 | gggggazgatzgtgggggggg              | sos |
| 1057 | gggggagctagctgggggggg              | sos |
| 1058 | ggttctttggcctgtct                  | s   |
| 1059 | ggttctttggcctgtct                  | s   |
| 1060 | ggttctttggccttatct                 | s   |

While CpG effects in mice are well characterized, information regarding the human system is limited. CpG phosphorothioate oligonucleotides with strong stimulatory activity in the mouse system show lower activity on human and other non-rodent immune cells. In the examples the development of a potent human CpG motif and the characterization of its effects and mechanisms of action on human primary B-cells is described. DNA containing this CpG motif strongly stimulated primary human B-cells to proliferate, to produce IL-6 and to express increased levels of CD86, CD40, CD54 and

MHC II. It increased DNA binding activity of the transcription factors NF $\kappa$ B and AP-1, as well as phosphorylation of the stress activated protein kinases JNK and p38, and the transcription factor ATF-2. B-cell signaling pathways activated by CpG DNA were different from those activated by the B-cell receptor which activated ERK and a different isoform of JNK, but did not activate p38 and ATF-2. In general the data on CpG DNA-initiated signal transduction are consistent with those obtained in mice (Hacker H., Mischak H., Miethke T., Liptay S., Schmid R., Sparwasser T., Heeg K., Lipford G. B., and Wagner H. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *Embo J* 17:6230, Yi A. K., and Krieg A. M. 1998. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J Immunol* 161:4493).

The preferred non-rodent motif is 5' TCGTCGTT 3'. Base exchanges within the most potent 8mer CpG motif (5' TCGTCGTT 3') diminished the activity of the oligonucleotide. The thymidines at the 5' and the 3' position of this motif were more important than the thymidine at the middle position. An adenine or guanosine at the middle position produced a decrease in the activity.

Of note, our studies demonstrate that one human CpG motif within a phosphodiester oligonucleotide (2080) is sufficient to produce the maximal effect, and that additional CpG motifs (2059) did not further enhance the activity. The oligonucleotide with the 8mer motif 5' TCG TCG TT 3' (2080) containing two CpG dinucleotides showed the highest activity in the studies. Replacement of the bases flanking the two CpG dinucleotides (5' position, middle position, 3' position) reduced the activity of this sequence. Both CpG dinucleotides within the 8mer CpG motif were required for the optimal activity (2108, 2106). Cytidine methylation of the CpG dinucleotides (2095) abolished the activity of 2080, while methylation of an unrelated cytidine (2094) did not. The addition of two CpG motifs into the sequence of 2080 resulting in 2059 did not further increase the activity of the phosphodiester oligonucleotide. The sequence of 2080 with a phosphorothioate backbone (2116) demonstrated less activity, suggesting that additional CpG motifs are preferred for a potent phosphorothioate oligonucleotide.

It has been discovered according to the invention that the immunostimulatory nucleic acids have dramatic immune stimulatory effects on human cells such as NK cells, B cells, and DCs *in vitro*. It has been demonstrated that that the *in vitro* assays used herein predict *in vivo* effectiveness as a vaccine adjuvant in non-rodent vertebrates 5 (Example 12), suggesting that immunostimulatory nucleic acids are effective therapeutic agents for human vaccination, cancer immunotherapy, asthma immunotherapy, general enhancement of immune function, enhancement of hematopoietic recovery following radiation or chemotherapy, and other immune modulatory applications.

Thus the immunostimulatory nucleic acids are useful in some aspects of the 10 invention as a prophylactic vaccine for the treatment of a subject at risk of developing an infection with an infectious organism or a cancer in which a specific cancer antigen has been identified or an allergy or asthma where the allergen or predisposition to asthma is known. The immunostimulatory nucleic acids can also be given without the antigen or allergen for shorter term protection against infection, allergy or cancer, and in this case 15 repeated doses will allow longer term protection. A subject at risk as used herein is a subject who has any risk of exposure to an infection causing pathogen or a cancer or an allergen or a risk of developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent is found or it may be a subject who through lifestyle or medical procedures is exposed to bodily 20 fluids which may contain infectious organisms or directly to the organism or even any subject living in an area where an infectious organism or an allergen has been identified. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular 25 antigen and the subject may be exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. A subject at risk of developing an allergy to asthma includes those subjects that have been identified as having an allergy or asthma but that don't have the active disease during the immunostimulatory nucleic acid treatment as well as subjects that are considered to be at risk of developing these diseases 30 because of genetic or environmental factors.

A subject at risk of developing a cancer is one who is who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic

abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins, or a subject who has previously been treated for cancer and is in apparent remission. When a subject at risk of 5 developing a cancer is treated with an antigen specific for the type of cancer to which the subject is at risk of developing and a immunostimulatory nucleic acid, the subject may be able to kill the cancer cells as they develop. If a tumor begins to form in the subject, the subject will develop a specific immune response against the tumor antigen.

In addition to the use of the immunostimulatory nucleic acids for prophylactic 10 treatment, the invention also encompasses the use of the immunostimulatory nucleic acids for the treatment of a subject having an infection, an allergy, asthma, or a cancer.

A subject having an infection is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The immunostimulatory nucleic acids can be used with an antigen to mount an antigen 15 specific systemic or mucosal immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. It is particularly important to develop effective vaccine strategies and treatments to protect the body's mucosal surfaces, which are the primary site of pathogenic entry.

20 A subject having an allergy is a subject that has or is at risk of developing an allergic reaction in response to an allergen. An allergy refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, conjunctivitis, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions.

25 Currently, allergic diseases are generally treated by the injection of small doses of antigen followed by subsequent increasing dosage of antigen. It is believed that this procedure induces tolerization to the allergen to prevent further allergic reactions. These methods, however, can take several years to be effective and are associated with the risk of side effects such as anaphylactic shock. The methods of the invention avoid these 30 problems.

Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by systemic or mucosal administration of

immunostimulatory nucleic acids are predominantly of a class called Th1 (examples are IL-12 and IFN- $\gamma$ ) and these induce both humoral and cellular immune responses. The types of antibodies associated with a Th1 response are generally more protective because they have high neutralization and opsonization capabilities. The other major type of 5 immune response, which is associated with the production of IL-4, IL-5 and IL-10 cytokines, is termed a Th2 immune response. Th2 responses involve predominately antibodies and these have less protective effect against infection and some Th2 isotypes (e.g., IgE) are associated with allergy. In general, it appears that allergic diseases are mediated by Th2 type immune responses while Th1 responses provide the best 10 protection against infection, although excessive Th1 responses are associated with autoimmune disease. Based on the ability of the immunostimulatory nucleic acids to shift the immune response in a subject from a Th2 (which is associated with production of IgE antibodies and allergy) to a Th1 response (which is protective against allergic reactions), an effective dose for inducing an immune response of a immunostimulatory 15 nucleic acid can be administered to a subject to treat or prevent an allergy.

Thus, the immunostimulatory nucleic acids have significant therapeutic utility in the treatment of allergic and non-allergic conditions such as asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including 20 IgE isotope switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN- $\gamma$  and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. Asthma refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated 25 with atopic or allergic symptoms.

A subject having a cancer is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; 30 intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid

cancer; and renal cancer, as well as other carcinomas and sarcomas. In one embodiment the cancer is hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, or colon carcinoma.

5 carcinoma.

A subject according to the invention is a non-rodent subject. A non-rodent subject shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, e.g., monkey, and fish (aquaculture species), e.g. salmon, but specifically excluding rodents such as rats and mice.

10 Thus, the invention can also be used to treat cancer and tumors in non human subjects. Cancer is one of the leading causes of death in companion animals (i.e., cats and dogs). Cancer usually strikes older animals which, in the case of house pets, have become integrated into the family. Forty-five % of dogs older than 10 years of age, are likely to succumb to the disease. The most common treatment options include surgery, 15 chemotherapy and radiation therapy. Others treatment modalities which have been used with some success are laser therapy, cryotherapy, hyperthermia and immunotherapy. The choice of treatment depends on type of cancer and degree of dissemination. Unless the malignant growth is confined to a discrete area in the body, it is difficult to remove only malignant tissue without also affecting normal cells.

20 Malignant disorders commonly diagnosed in dogs and cats include but are not limited to lymphosarcoma, osteosarcoma, mammary tumors, mastocytoma, brain tumor, melanoma, adenosquamous carcinoma, carcinoid lung tumor, bronchial gland tumor, bronchiolar adenocarcinoma, fibroma, myxochondroma, pulmonary sarcoma, neurosarcoma, osteoma, papilloma, retinoblastoma, Ewing's sarcoma, Wilm's tumor, Burkitt's lymphoma, microglioma, neuroblastoma, osteoclastoma, oral neoplasia, 25 fibrosarcoma, osteosarcoma and rhabdomyosarcoma. Other neoplasias in dogs include genital squamous cell carcinoma, transmissible venereal tumor, testicular tumor, seminoma, Sertoli cell tumor, hemangiopericytoma, histiocytoma, chloroma (granulocytic sarcoma), corneal papilloma, corneal squamous cell carcinoma, 30 hemangiosarcoma, pleural mesothelioma, basal cell tumor, thymoma, stomach tumor, adrenal gland carcinoma, oral papillomatosis, hemangioendothelioma and cystadenoma. Additional malignancies diagnosed in cats include follicular lymphoma, intestinal

lymphosarcoma, fibrosarcoma and pulmonary squamous cell carcinoma. The ferret, an ever-more popular house pet is known to develop insulinoma, lymphoma, sarcoma, neuroma, pancreatic islet cell tumor, gastric MALT lymphoma and gastric adenocarcinoma.

- 5        Neoplasias affecting agricultural livestock include leukemia, hemangiopericytoma and bovine ocular neoplasia (in cattle); preputial fibrosarcoma, ulcerative squamous cell carcinoma, preputial carcinoma, connective tissue neoplasia and mastocytoma (in horses); hepatocellular carcinoma (in swine); lymphoma and pulmonary adenomatosis (in sheep); pulmonary sarcoma, lymphoma, Rous sarcoma,
- 10      reticul endotheliosis, fibrosarcoma, nephroblastoma, B-cell lymphoma and lymphoid leukosis (in avian species); retinoblastoma, hepatic neoplasia, lymphosarcoma (lymphoblastic lymphoma), plasmacytoid leukemia and swimbladder sarcoma (in fish), caseous lymphadenitis (CLA): chronic, infectious, contagious disease of sheep and goats caused by the bacterium *Corynebacterium pseudotuberculosis*, and contagious lung
- 15      tumor of sheep caused by jaagsiekte.

The subject is exposed to the antigen. As used herein, the term exposed to refers to either the active step of contacting the subject with an antigen or the passive exposure of the subject to the antigen *in vivo*. Methods for the active exposure of a subject to an antigen are well-known in the art. In general, an antigen is administered directly to the subject by any means such as intravenous, intramuscular, oral, transdermal, mucosal, intranasal, intratracheal, or subcutaneous administration. The antigen can be administered systemically or locally. Methods for administering the antigen and the immunostimulatory nucleic acid are described in more detail below. A subject is passively exposed to an antigen if an antigen becomes available for exposure to the immune cells in the body. A subject may be passively exposed to an antigen, for instance, by entry of a foreign pathogen into the body or by the development of a tumor cell expressing a foreign antigen on its surface.

The methods in which a subject is passively exposed to an antigen can be particularly dependent on timing of administration of the immunostimulatory nucleic acid. For instance, in a subject at risk of developing a cancer or an infectious disease or an allergic or asthmatic response, the subject may be administered the immunostimulatory nucleic acid on a regular basis when that risk is greatest, i.e., during

allergy season or after exposure to a cancer causing agent. Additionally the immunostimulatory nucleic acid may be administered to travelers before they travel to foreign lands where they are at risk of exposure to infectious agents. Likewise the immunostimulatory nucleic acid may be administered to soldiers or civilians at risk of 5 exposure to biowarfare to induce a systemic or mucosal immune response to the antigen when and if the subject is exposed to it.

An antigen as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, 10 glycolipids, carbohydrates, viruses and viral extracts and multicellular organisms such as parasites and allergens. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens, and allergens.

15 A cancer antigen as used herein is a compound, such as a peptide or protein, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 20 1994, *Cancer Research*, 54:1055, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include but are not limited to antigens that are recombinantly expressed, an immunogenic portion of, or a whole tumor or cancer. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

25 A microbial antigen as used herein is an antigen of a microorganism and includes but is not limited to virus, bacteria, parasites, and fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar 30 to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to those of ordinary skill in the art.

Examples of viruses that have been found in humans include but are not limited to: *Retroviridae* (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g. strains that cause gastroenteritis); *Togaviridae* (e.g. equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g. coronaviruses); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Coronaviridae* (e.g. coronaviruses); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g. ebola viruses); *Paramyxoviridae* (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g. influenza viruses); *Bunyaviridae* (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g. reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to, *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to, *Helicobacter pyloris*, *Borelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria* sps (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus*

*bovis*, *Streptococcus* (anaerobic spp.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp., *Enterococcus* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *corynebacterium diphtheriae*, *corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelii*.

5 Examples of fungi include *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*.

10 Other infectious organisms (i.e., protists) include *Plasmodium* spp. such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax* and *Toxoplasma gondii*. Blood-borne and/or tissues parasites include *Plasmodium* spp., *Babesia microti*, *Babesia divergens*, *Leishmania tropica*, *Leishmania* spp., *15 Leishmania braziliensis*, *Leishmania donovani*, *Trypanosoma gambiense* and *Trypanosoma rhodesiense* (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Toxoplasma gondii*.

15 Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

20 Although many of the microbial antigens described above relate to human disorders, the invention is also useful for treating other nonhuman vertebrates. Nonhuman vertebrates are also capable of developing infections which can be prevented or treated with the Immunostimulatory nucleic acids disclosed herein. For instance, in 25 addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals.

25 As used herein, the term treat, treated, or treating when used with respect to an infectious disease refers to a prophylactic treatment which increases the resistance of a subject (a subject at risk of infection) to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen as well as a treatment after the subject (a subject who has been infected) has become infected in

order to fight the infection, e.g., reduce or eliminate the infection or prevent it from becoming worse.

Many vaccines for the treatment of non-human vertebrates are disclosed in Bennett, K. *Compendium of Veterinary Products*, 3rd ed. North American Compendiums, Inc., 1995. As discussed above, antigens include infectious microbes such as virus, parasite, bacteria and fungi and fragments thereof, derived from natural sources or synthetically. Infectious viruses of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. This group of retroviruses includes both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroups of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV)) and C-type group B (including feline leukemia virus (FeLV), gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (RV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of other RNA viruses that are antigens in vertebrate animals include, but are not limited to, members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus, and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picornaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A virus, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine

enteroviruses, Porcine enteroviruses , the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Aphtovirus (Foot and Mouth disease (FMDV); the family Picornaviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus); the family Rhabdoviridae, including the genus Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies virus), fish Rhabdoviruses, and two probable Rhabdoviruses (Marburg virus and Ebola virus); the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family Coronaviridae, including Infectious Bronchitis Virus (IBV), Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that are antigens in vertebrate animals include, but are not limited to, the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia), the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus), the genus Capripoxvirus (sheppox, goatpox), the genus Suipoxvirus (Swinepox), the genus Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine and monkeys); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus atelus, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A,B,C,D,E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotrophic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

Each of the foregoing lists is illustrative, and is not intended to be limiting. In addition to the use of the immunostimulatory nucleic acids to induce an antigen specific immune response in humans, the methods of the preferred embodiments

are particularly well suited for treatment of birds such as hens, chickens, turkeys, ducks, geese, quail, and pheasant. Birds are prime targets for many types of infections.

Hatching birds are exposed to pathogenic microorganisms shortly after birth. Although these birds are initially protected against pathogens by maternal derived 5 antibodies, this protection is only temporary, and the bird's own immature immune system must begin to protect the bird against the pathogens. It is often desirable to prevent infection in young birds when they are most susceptible. It is also desirable to prevent against infection in older birds, especially when the birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the 10 Immunostimulatory nucleic acid and the non-nucleic acid adjuvant of the invention to birds to enhance an antigen-specific immune response when antigen is present.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yuasa et al., 1979, Avian Dis. 23:366-385). Since 15 that time, CIAV has been detected in commercial poultry in all major poultry producing countries (van Bulow et al., 1991, pp.690-699) in Diseases of Poultry, 9th edition, Iowa State University Press).

CIAV infection results in a clinical disease, characterized by anemia, hemorrhage and immunosuppression, in young susceptible chickens. Atrophy of the 20 thymus and of the bone marrow and consistent lesions of CIAV-infected chickens are also characteristic of CIAV infection. Lymphocyte depletion in the thymus, and occasionally in the bursa of Fabricius, results in immunosuppression and increased susceptibility to secondary viral, bacterial, or fungal infections which then complicate the course of the disease. The immunosuppression may cause aggravated disease after 25 infection with one or more of Marek's disease virus (MDV), infectious bursal disease virus, reticuloendotheliosis virus, adenovirus, or reovirus. It has been reported that pathogenesis of MDV is enhanced by CIAV (DeBoer et al., 1989, p. 28 In Proceedings of the 38th Western Poultry Diseases Conference, Tempe, Ariz.). Further, it has been reported that CIAV aggravates the signs of infectious bursal disease (Rosenberger et al., 30 1989, Avian Dis. 33:707-713). Chickens develop an age resistance to experimentally induced disease due to CAA. This is essentially complete by the age of 2 weeks, but older birds are still susceptible to infection (Yuasa, N. et al., 1979 supra; Yuasa, N. et al.,

Arian Diseases 24, 202-209, 1980). However, if chickens are dually infected with CAA and an immunosuppressive agent (IBDV, MDV etc.), age resistance against the disease is delayed (Yuasa, N. et al., 1979 and 1980 supra; Bulow von V. et al., J. Veterinary Medicine 33, 93-116, 1986). Characteristics of CIAV that may potentiate disease  
5 transmission include high resistance to environmental inactivation and some common disinfectants. The economic impact of CIAV infection on the poultry industry is clear from the fact that 10% to 30% of infected birds in disease outbreaks die.

Vaccination of birds, like other vertebrate animals can be performed at any age. Normally, vaccinations are performed at up to 12 weeks of age for a live microorganism  
10 and between 14-18 weeks for an inactivated microorganism or other type of vaccine. For in ovo vaccination, vaccination can be performed in the last quarter of embryo development. The vaccine may be administered subcutaneously, by spray, orally, intraocularly, intratracheally, nasally, or by other mucosal delivery methods described herein. Thus, the immunostimulatory nucleic acids of the invention can be administered  
15 to birds and other non-human vertebrates using routine vaccination schedules and the antigen can be administered after an appropriate time period as described herein.

Cattle and livestock are also susceptible to infection. Diseases which affect these animals can produce severe economic losses, especially amongst cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses,  
20 pigs, sheep, and goats.

Cows can be infected by bovine viruses. Bovine viral diarrhea virus (BVDV) is a small enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although, Pestiviruses were previously classified in the Togaviridae family, some studies have  
25 suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups (Francki, et al., 1991).

BVDV, which is an important pathogen of cattle can be distinguished, based on cell culture analysis, into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes. The NCP biotype is more widespread although both biotypes can be found in cattle. If a  
30 pregnant cow becomes infected with an NCP strain, the cow can give birth to a persistently infected and specifically immunotolerant calf that will spread virus during its lifetime. The persistently infected cattle can succumb to mucosal disease and both

biotypes can then be isolated from the animal. Clinical manifestations can include abortion, teratogenesis, and respiratory problems, mucosal disease and mild diarrhea. In addition, severe thrombocytopenia, associated with herd epidemics, that may result in the death of the animal has been described and strains associated with this disease seem 5 more virulent than the classical BVDVs.

Equine herpes viruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal disease. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and 10 central nervous system disorders. Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts for 8 to 10 days. Immunologically experienced mares may be re-infected via the respiratory tract without disease becoming apparent, so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of either sex at any 15 age, leading to lack of co-ordination, weakness and posterior paralysis (Telford, E. A. R. et al., Virology 189, 304-316, 1992). Other EHV's include EHV-2, or equine cytomegalovirus, EHV-3, equine coital exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

Sheep and goats can be infected by a variety of dangerous microorganisms 20 including visna-maedi.

Primates such as monkeys, apes and macaques can be infected by simian immunodeficiency virus. Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 36:1538-1541; Desrosiers et al. PNAS USA (1989) 86:6353-6357; 25 Murphrey-Corb et al. (1989) Science 246:1293-1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

Cats, both domestic and wild, are susceptible to infection with a variety of 30 microorganisms. For instance, feline infectious peritonitis is a disease which occurs in both domestic and wild cats, such as lions, leopards, cheetahs, and jaguars. When it is desirable to prevent infection with this and other types of pathogenic organisms in cats,

the methods of the invention can be used to vaccinate cats to protect them against infection.

Domestic cats may become infected with several retroviruses, including but not limited to feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type 5 Concornavirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immune mediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has 10 been more particularly associated with immunosuppressive properties.

The discovery of feline T-lymphotropic lentivirus (also referred to as feline immunodeficiency) was first reported in Pedersen et al. (1987) Science 235:790-793. Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 15 49:1246-1258; and Ackley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. USA 86:2448-2452 and 86:4355-4360.

Feline infectious peritonitis (FIP) is a sporadic disease occurring unpredictably in domestic and wild Felidae. While FIP is primarily a disease of domestic cats, it has been 20 diagnosed in lions, mountain lions, leopards, cheetahs, and the jaguar. Smaller wild cats that have been afflicted with FIP include the lynx and caracal, sand cat, and pallas cat. In domestic cats, the disease occurs predominantly in young animals, although cats of all ages are susceptible. A peak incidence occurs between 6 and 12 months of age. A decline in incidence is noted from 5 to 13 years of age, followed by an increased 25 incidence in cats 14 to 15 years old.

Viral, bacterial, and parasitic diseases in fin-fish, shellfish or other aquatic life forms pose a serious problem for the aquaculture industry. Owing to the high density of animals in the hatchery tanks or enclosed marine farming areas, infectious diseases may eradicate a large proportion of the stock in, for example, a fin-fish, shellfish, or other 30 aquatic life forms facility. Prevention of disease is a more desired remedy to these threats to fish than intervention once the disease is in progress. Vaccination of fish is the only preventative method which may offer long-term protection through immunity.

Nucleic acid based vaccinations are described in US Patent No. 5,780,448 issued to Davis.

The fish immune system has many features similar to the mammalian immune system, such as the presence of B cells, T cells, lymphokines, complement, and 5 immunoglobulins. Fish have lymphocyte subclasses with roles that appear similar in many respects to those of the B and T cells of mammals. Vaccines can be administered by immersion or orally.

Aquaculture species include but are not limited to fin-fish, shellfish, and other aquatic animals. Fin-fish include all vertebrate fish, which may be bony or cartilaginous 10 fish, such as, for example, salmonids, carp, catfish, yellowtail, seabream, and seabass. Salmonids are a family of fin-fish which include trout (including rainbow trout), salmon, and Arctic char. Examples of shellfish include, but are not limited to, clams, lobster, shrimp, crab, and oysters. Other cultured aquatic animals include, but are not limited to eels, squid, and octopi.

15 Polypeptides of viral aquaculture pathogens include but are not limited to glycoprotein (G) or nucleoprotein (N) of viral hemorrhagic septicemia virus (VHSV); G or N proteins of infectious hematopoietic necrosis virus (IHNV); VP1, VP2, VP3 or N structural proteins of infectious pancreatic necrosis virus (IPNV); G protein of spring viremia of carp (SVC); and a membrane-associated protein, tegumin or capsid protein or 20 glycoprotein of channel catfish virus (CCV).

Typical parasites infecting horses are *Gasterophilus* spp.; *Eimeria leuckarti*, *Giardia* spp.; *Tritrichomonas equi*; *Babesia* spp. (RBC's), *Theileria equi*; *Trypanosoma* spp.; *Klossiella equi*; *Sarcocystis* spp.

Typical parasites infecting swine include *Eimeria bebliecki*, *Eimeria scabra*, 25 *Isospora suis*, *Giardia* spp.; *Balantidium coli*, *Entamoeba histolytica*; *Toxoplasma gondii* and *Sarcocystis* spp., and *Trichinella spiralis*.

The major parasites of dairy and beef cattle include *Eimeria* spp., *Cryptosporidium* sp., *Giardia* spp.; *Toxoplasma gondii*; *Babesia bovis* (RBC), *Babesia bigemina* (RBC), *Trypanosoma* spp. (plasma), *Theileria* spp. (RBC); *Theileria parva* 30 (lymphocytes); *Tritrichomonas foetus*; and *Sarcocystis* spp.

The major parasites of raptors include *Trichomonas gallinae*; Coccidia (*Eimeria* spp.); *Plasmodium relictum*, *Leucocytozoon danilewskyi* (owls), *Haemoproteus* spp.,

*Trypanosoma* spp.; *Histomonas*; *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, *Giardia*, *Eimeria*; *Toxoplasma*.

Typical parasites infecting sheep and goats include *Eimeria* spp., *Cryptosporidium* sp., *Giardia* sp.; *Toxoplasma gondii*; *Babesia* spp. (RBC),  
5 *Trypanosoma* spp. (plasma), *Theileria* spp. (RBC); and *Sarcocystis* spp.

Typical parasitic infections in poultry include coccidiosis caused by *Eimeria acervulina*, *E. necatrix*, *E. tenella*, *Isospora* spp. and *Eimeria truncata*; histomoniasis, caused by *Histomonas meleagridis* and *Histomonas gallinarum*; trichomoniasis caused by *Trichomonas gallinae*; and hexamitiasis caused by *Hexamita meleagridis*. Poultry  
10 can also be infected *Emeria maxima*, *Emeria meleagridis*, *Eimeria adenoeides*, *Eimeria meleagrinitis*, *Cryptosporidium*, *Eimeria brunetti*, *Emeria adenoeides*, *Leucocytozoon* spp., *Plasmodium* spp., *Hemoproteus meleagridis*, *Toxoplasma gondii* and *Sarcocystis*.

The methods of the invention can also be applied to the treatment and/or prevention of parasitic infection in dogs, cats, birds, fish and ferrets. Typical parasites of  
15 birds include *Trichomonas gallinae*; *Eimeria* spp., *Isospora* spp., *Giardia*, *Cryptosporidium*, *Sarcocystis* spp., *Toxoplasma gondii*, *Haemoproteus/Parahaemoproteus*, *Plasmodium* spp., *Leucocytozoon/Akiba*, *Atoxoplasma*, *Trypanosoma* spp. Typical parasites infecting dogs include *Trichinella spiralis*; *Isopora* spp., *Sarcocystis* spp., *Cryptosporidium* spp., *Hammondia* spp., *Giardia*  
20 *duodenalis (canis)*; *Balantidium coli*, *Entamoeba histolytica*; *Hepatozoon canis*; *Toxoplasma gondii*, *Trypanosoma cruzi*; *Babesia canis*; *Leishmania amastigotes*; *Neospora caninum*.

Typical parasites infecting feline species include *Isospora* spp., *Toxoplasma gondii*, *Sarcocystis* spp., *Hammondia hammondi*, *Besnoitia* spp., *Giardia* spp.;  
25 *Entamoeba histolytica*; *Hepatozoon canis*, *Cytauxzoon* sp., *Cytauxzoon* sp., *Cytauxzoon* sp. (red cells, RE cells).

Typical parasites infecting fish include *Hexamita* spp., *Eimeria* spp.; *Cryptobia* spp., *Nosema* spp., *Myxosoma* spp., *Chilodonella* spp., *Trichodina* spp.; *Plistophora* spp., *Myxosoma Henneguya*; *Costia* spp., *Ichthyophthirius* spp., and *Oodinium* spp.

30 Typical parasites of wild mammals include *Giardia* spp. (carnivores, herbivores), *Isospora* spp. (carnivores), *Eimeria* spp. (carnivores, herbivores); *Theileria* spp. (herbivores), *Babesia* spp. (carnivores, herbivores), *Trypanosoma* spp. (carnivores,

herbivores); *Schistosoma* spp. (herbivores); *Fasciola hepatica* (herbivores), *Fascioloides magna* (herbivores), *Fasciola gigantica* (herbivores), *Trichinella spiralis* (carnivores, herbivores).

Parasitic infections in zoos can also pose serious problems. Typical parasites of  
5 the bovidae family (blesbok, antelope, banteng, eland, gaur, impala, klipspringer, kudu, gazelle) include *Eimeria* spp. Typical parasites in the pinnipedae family (seal, sea lion) include *Eimeria phocae*. Typical parasites in the camelidae family (camels, llamas) include *Eimeria* spp. Typical parasites of the giraffidae family (giraffes) include *Eimeria* spp. Typical parasites in the elephantidae family (African and Asian) include *Fasciola*  
10 spp. Typical parasites of lower primates (chimpanzees, orangutans, apes, baboons, macaques, monkeys) include *Giardia* sp.; *Balantidium coli*, *Entamoeba histolytica*, *Sarcocystis* spp., *Toxoplasma gondii*; *Plasmodium* spp. (RBC), *Babesia* spp. (RBC), *Trypanosoma* spp. (plasma), *Leishmania* spp. (macrophages).

Polypeptides of bacterial pathogens include but are not limited to an  
15 iron-regulated outer membrane protein, (IOMP), an outer membrane protein (OMP), and an A-protein of *Aeromonis salmonicida* which causes furunculosis, p57 protein of *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD), major surface associated antigen (msa), a surface expressed cytotoxin (mpr), a surface expressed hemolysin (ish), and a flagellar antigen of *Yersinia*; an extracellular protein  
20 (ECP), an iron-regulated outer membrane protein (IOMP), and a structural protein of *Pasteurelllosis*; an OMP and a flagellar protein of *Vibrosis anguillarum* and *V. ordalii*; a flagellar protein, an OMP protein, aroA, and purA of *Edwardsiellosis ictaluri* and *E. tarda*; and surface antigen of *Ichthyophthirius*; and a structural and regulatory protein of *Cytophaga columnari*; and a structural and regulatory protein of *Rickettsia*.  
25 Polypeptides of a parasitic pathogen include but are not limited to the surface antigens of *Ichthyophthirius*.

An allergen refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g.  
30 penicillin). Examples of natural, animal and plant allergens include but are not limited to proteins specific to the following genera: *Canine* (*Canis familiaris*); *Dermatophagoides* (e.g. *Dermatophagoides farinae*); *Felis* (*Felis domesticus*); *Ambrosia* (*Ambrosia*

- artemisiifolia; Lolium* (e.g. *Lolium perenne* or *Lolium multiflorum*); *Cryptomeria* (*Cryptomeria japonica*); *Alternaria* (*Alternaria alternata*); *Alder*; *Alnus* (*Alnus glutinosa*); *Betula* (*Betula verrucosa*); *Quercus* (*Quercus alba*); *Olea* (*Olea europaea*); *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g. *Plantago lanceolata*); *Parietaria* (e.g. 5 *Parietaria officinalis* or *Parietaria judaica*); *Blattella* (e.g. *Blattella germanica*); *Apis* (e.g. *Apis multiflorum*); *Cupressus* (e.g. *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); *Juniperus* (e.g. *Juniperus sabinaoides*, *Juniperus virginiana*, *Juniperus communis* and *Juniperus ashei*); *Thuya* (e.g. *Thuya orientalis*); *Chamaecyparis* (e.g. *Chamaecyparis obtusa*); *Periplaneta* (e.g. *Periplaneta americana*); 10 *Agropyron* (e.g. *Agropyron repens*); *Secale* (e.g. *Secale cereale*); *Triticum* (e.g. *Triticum aestivum*); *Dactylis* (e.g. *Dactylis glomerata*); *Festuca* (e.g. *Festuca elatior*); *Poa* (e.g. *Poa pratensis* or *Poa compressa*); *Avena* (e.g. *Avena sativa*); *Holcus* (e.g. *Holcus lanatus*); *Anthoxanthum* (e.g. *Anthoxanthum odoratum*); *Arrhenatherum* (e.g. 15 *Arrhenatherum elatius*); *Agrostis* (e.g. *Agrostis alba*); *Phleum* (e.g. *Phleum pratense*); *Phalaris* (e.g. *Phalaris arundinacea*); *Paspalum* (e.g. *Paspalum notatum*); *Sorghum* (e.g. *Sorghum halepensis*); and *Bromus* (e.g. *Bromus inermis*).

The antigen may be an antigen that is encoded by a nucleic acid vector or it may be not encoded in a nucleic acid vector. In the former case the nucleic acid vector is administered to the subject and the antigen is expressed *in vivo*. In the latter case the 20 antigen may be administered directly to the subject. An antigen not encoded in a nucleic acid vector as used herein refers to any type of antigen that is not a nucleic acid. For instance, in some aspects of the invention the antigen not encoded in a nucleic acid vector is a polypeptide. Minor modifications of the primary amino acid sequences of polypeptide antigens may also result in a polypeptide which has substantially equivalent 25 antigenic activity as compared to the unmodified counterpart polypeptide. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as antigenicity still exists. The polypeptide may be, for example, a viral polypeptide.

The term substantially purified as used herein refers to a polypeptide which is 30 substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify viral or bacterial polypeptides using standard techniques for protein purification. The substantially pure polypeptide

will often yield a single major band on a non-reducing polyacrylamide gel. In the case of partially glycosylated polypeptides or those that have several start codons, there may be several bands on a non-reducing polyacrylamide gel, but these will form a distinctive pattern for that polypeptide. The purity of the viral or bacterial polypeptide can also be  
5 determined by amino-terminal amino acid sequence analysis. Other types of antigens not encoded by a nucleic acid vector such as polysaccharides, small molecule, mimics etc are described above, and included within the invention.

The invention also utilizes polynucleotides encoding the antigenic polypeptides. It is envisioned that the antigen may be delivered to the subject in a nucleic acid  
10 molecule which encodes for the antigen such that the antigen must be expressed *in vivo*. Such antigens delivered to the subject in a nucleic acid vector are referred to as antigens encoded by a nucleic acid vector. The nucleic acid encoding the antigen is operatively linked to a gene expression sequence which directs the expression of the antigen nucleic acid within a eukaryotic cell. The gene expression sequence is any regulatory nucleotide  
15 sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the antigen nucleic acid to which it is operatively linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes:  
20 hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase, b-actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the cytomegalovirus (CMV), simian virus (e.g., SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long  
25 terminal repeats (LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein  
30 promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

- In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will
- 5 include a promoter region which includes a promoter sequence for transcriptional control of the operably joined antigen nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

The antigen nucleic acid is operatively linked to the gene expression sequence. As used herein, the antigen nucleic acid sequence and the gene expression sequence are

10 said to be operably linked when they are covalently linked in such a way as to place the expression or transcription and/or translation of the antigen coding sequence under the influence or control of the gene expression sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the antigen sequence and if the nature of the linkage between the

15 two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the antigen sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to an antigen nucleic acid sequence if the gene expression sequence were capable of

20 effecting transcription of that antigen nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

The antigen nucleic acid of the invention may be delivered to the immune system alone or in association with a vector. In its broadest sense, a vector is any vehicle capable of facilitating the transfer of the antigen nucleic acid to the cells of the immune

25 system so that the antigen can be expressed and presented on the surface of the immune cell. The vector generally transports the nucleic acid to the immune cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. The vector optionally includes the above-described gene expression sequence to enhance expression of the antigen nucleic acid in immune cells. In general, the vectors

30 useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antigen nucleic acid sequences. Viral vectors are a

preferred type of vector and include, but are not limited to, nucleic acid sequences from the following viruses: retrovirus, such as Moloney murine leukemia virus, Harvey murine sarcoma virus, murine mammary tumor virus, and Rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr 5 viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic 10 viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency 15 transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., 20 Gene Transfer and Expression, A Laboratory Manual W.H. Freeman C.O., New York (1990) and Murry, E.J. Methods in Molecular Biology, vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

A preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be 25 replication -deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, 30 thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than

100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA.

It has recently been discovered that gene carrying plasmids can be delivered to the immune system using bacteria. Modified forms of bacteria such as *Salmonella* can be transfected with the plasmid and used as delivery vehicles. The bacterial delivery vehicles can be administered to a host subject orally or by other administration means. The bacteria deliver the plasmid to immune cells, e.g. B cells, dendritic cells, likely by passing through the gut barrier. High levels of immune protection have been established using this methodology. Such methods of delivery are useful for the aspects of the invention utilizing systemic delivery of antigen, Immunostimulatory nucleic acid and/or other therapeutic agent.

Thus, the immunostimulatory nucleic acids are useful as vaccine adjuvants. It was previously established that CpG oligonucleotides are excellent vaccine adjuvants. It was also demonstrated, however, that CpG ODN which are superb vaccine adjuvants in mice are not the preferred adjuvants in non-rodent animals. In order to identify the best immunostimulatory nucleic acids for use as a vaccine adjuvant in humans and other non-rodent animals, *in vivo* screening of different nucleic acids for this purpose was conducted. Several *in vitro* assays were evaluated in mice for their predictive value of adjuvant activity *in vivo* in mice. During the course of this study, an *in vitro* test that is predictive of *in vivo* efficacy was identified. It was discovered, rather surprisingly, that

both B cell and NK cell activation correlated particularly well with the ability of an immunostimulatory nucleic acid to enhance an *in vivo* immune response against an antigen.

The good predictive value of B cell activation for *in vivo* vaccine adjuvant activity is most likely linked to the central role of B cells in the establishment of a specific immune response. Polyclonal proliferation of B cells (induced by immunostimulatory nucleic acids) increases the likelihood of an antigen specific B cell/T helper cell match. Furthermore, enhanced expression of the co-stimulatory molecule CD86 on polyclonally expanded B cells activates antigen specific T helper cells. B cells also increase their CD40 expression in response to immunostimulatory nucleic acids improving the capability of CD40L expressing activated T helper cells to stimulate B cells. Increased ICAM-1 synthesis on B cells facilitates the cell to cell contact. Thus, the activation status of polyclonal B cells plays a critical role during the initiation of a specific antibody response.

The contribution of NK cell activity for the establishment of specific antibodies was, however, surprising. NK cells are part of the innate immune system and as such are involved in the first line of defense against pathogens. Most likely the cytokine pattern produced by NK cells upon activation is closely related to the initiation of a specific immune response. Thus, in one aspect the invention relates to a method of identifying an adjuvant, by detecting NK cell activation. The NK cell activation assay may be carried out as described in the Examples below or using other known NK cell activity assays. It is preferred, however that a mixed cell population such as PBMC be used because of the likelihood that NK cell activation is an indirect effect. The assay is preferably useful for identifying immunostimulatory nucleic acids which are useful as adjuvants in human and other non-rodent animals.

Cytokine induction was also identified as an important predictor of *in vivo* adjuvant activity. As there is a 2 log higher endotoxin sensitivity of human than mouse primary monocytes, some caution, however, is required to avoid endotoxin contamination of immunostimulatory nucleic acids used for testing in the human system (Hartmann G., and Krieg A. M. 1999. *Gene Therapy* 6:893). Since TNF- $\alpha$ , IL-6 and IL-12 are produced by human monocytes in response to even low amounts of endotoxin, their value for high throughput *in vitro* screening assays is limited. On the other hand,

human B cells and NK cells show only minor activation by endotoxin and thus are far more useful in testing for immunostimulatory activity.

- Stimulation of cellular function in either NK or B cells (i.e., lytic activity, proliferation) requires a stronger immunostimulatory nucleic acid than the induction of 5 activation markers at their surface (CD69, CD86). For both cell types, the use of cell surface activation markers showed a higher nonspecific background attributable to the phosphorothioate backbone compared to the functional assays. This high sensitivity of surface markers requires the use of low immunostimulatory nucleic acid concentrations for optimal discrimination between immunostimulatory nucleic acid of similar activity.
- 10 Thus, the use of surface markers allows the comparison of immunostimulatory nucleic acids with weak activity, while functional assays are preferred for comparing immunostimulatory nucleic acids with high activity. It is of note that the optimal immunostimulatory nucleic acid concentrations for stimulating B cells and NK cells differ. While 0.6 µg/ml ODN is already maximal to stimulate B cells, optimal NK cell 15 activation may require 6 µg/ml ODN. Both B cell activation and NK cell functional activity were measured within freshly isolated PBMC. It was previously found that highly purified human primary B cells are activated by CpG DNA. The existence of a direct effect of CpG DNA on NK cells is less clear, and a secondary mechanism mediated by another cell type within PBMC might contribute to CpG-induced functional 20 activity of NK cells.

The nucleic acids of the invention may be administered to a subject with an anti-microbial agent. An anti-microbial agent, as used herein, refers to a naturally-occurring or synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the invention will 25 depend upon the type of microorganism with which the subject is infected or at risk of becoming infected. Anti-microbial agents include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as “anti-infective agent”, “anti-bacterial agent”, “anti-viral agent”, “anti-fungal agent”, “anti-parasitic agent” and “parasiticide” have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial 30 agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Antibiotics are low molecular weight molecules

which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more bacterial functions or structures which are specific for the microorganism and which are not present in host cells. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasite agents kill or inhibit parasites.

Examples of anti-parasitic agents, also referred to as parasiticides useful for human administration include but are not limited to albendazole, amphotericin B, 10 benznidazole, bithionol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, diloxanide furoate, eflornithine, furazolidone, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate, melarsoprol, metrifonate, metronidazole, niclosamide, nifurtimox, oxamniquine, paromomycin, pentamidine isethionate, piperazine, 15 praziquantel, primaquine phosphate, proguanil, pyrantel pamoate, pyrimethamine-sulfonamides, pyrimethamine-sulfadoxine, quinacrine HCl, quinine sulfate, quinidine gluconate, spiramycin, stibogluconate sodium (sodium antimony gluconate), suramin, tetracycline, doxycycline, thiabendazole, tinidazole, trimethoprim-sulfamethoxazole, and tryparsamide some of which are used alone or in combination with others.

Parasiticides used in non-human subjects include piperazine, 20 diethylcarbamazine, thiabendazole, fenbendazole, albendazole, oxfendazole, oxibendazole, febantel, levamisole, pyrantel tartrate, pyrantel pamoate, dichlorvos, ivermectin, doramectic, milbemycin oxime, ivermectin, moxidectin, N-butyl chloride, toluene, hygromycin B thiacetarsemide sodium, melarsomine, praziquantel, epsiprantel, 25 benzimidazoles such as fenbendazole, albendazole, oxfendazole, clorsulon, albendazole, amprolium; decoquinate, lasalocid, monensin sulfadimethoxine; sulfamethazine, sulfaquinoxaline, metronidazole.

Parasiticides used in horses include mebendazole, oxfendazole, febantel, 30 pyrantel, dichlorvos, trichlorfon, ivermectin, piperazine; for *S. westeri*: ivermectin, benzimidazoles such as thiabendazole, cambendazole, oxibendazole and fenbendazole. Useful parasiticides in dogs include milbemycin oxime, ivermectin, pyrantel pamoate and the combination of ivermectin and pyrantel. The treatment of parasites in swine can

include the use of levamisole, piperazine, pyrantel, thiabendazole, dichlorvos and fenbendazole. In sheep and goats anthelmintic agents include levamisole or ivermectin. Caparsolate has shown some efficacy in the treatment of *D. immitis* (heartworm) in cats.

Antibacterial agents kill or inhibit the growth or function of bacteria. A large 5 class of antibacterial agents is antibiotics. Antibiotics, which are effective for killing or inhibiting a wide range of bacteria, are referred to as broad spectrum antibiotics. Other types of antibiotics are predominantly effective against the bacteria of the class gram-positive or gram-negative. These types of antibiotics are referred to as narrow spectrum antibiotics. Other antibiotics which are effective against a single organism or disease 10 and not against other types of bacteria, are referred to as limited spectrum antibiotics. Antibacterial agents are sometimes classified based on their primary mode of action. In general, antibacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors.

15 Anti-bacterial agents useful in the invention include but are not limited to natural penicillins, semi-synthetic penicillins, clavulanic acid, cephalosporins, bacitracin, ampicillin, carbenicillin, oxacillin, azlocillin, mezlocillin, piperacillin, methicillin, dicloxacillin, nafcillin, cephalothin, cephapirin, cephalexin, cefamandole, cefaclor, cefazolin, cefuroxine, cefoxitin, cefotaxime, cefsulodin, cefetamet, cefixime, ceftriaxone, 20 cefoperazone, ceftazidime, moxalactam, carbapenems, imipenems, monobactems, euztreonam, vancomycin, polymyxin, amphotericin B, nystatin, imidazoles, clotrimazole, miconazole, ketoconazole, itraconazole, fluconazole, rifampins, ethambutol, tetracyclines, chloramphenicol, macrolides, aminoglycosides, streptomycin, kanamycin, tobramycin, amikacin, gentamicin, tetracycline, minocycline, doxycycline, 25 chlortetracycline, erythromycin, roxithromycin, clarithromycin, oleandomycin, azithromycin, chloramphenicol, quinolones, co-trimoxazole, norfloxacin, ciprofloxacin, enoxacin, nalidixic acid, temafloxacin, sulfonamides, gantrisin, and trimethoprim; Acedapsone ; Acetosulfone Sodium; Alamecin; Alexidine; Amdinocillin; Amdinocillin Pivoxil; Amicycline; Amifloxacin; Amifloxacin Mesylate; Amikacin; Amikacin Sulfate; 30 Aminosalicylic acid; Aminosalicylate sodium; Amoxicillin; Amphomycin; Ampicillin; Ampicillin Sodium; Apalcillin Sodium; Apramycin; Aspartocin; Astromicin Sulfate; Avilamycin; Avoparcin; Azithromycin; Azlocillin; Azlocillin Sodium; Bacampicillin

- Hydrochloride; Bacitracin; Bacitracin Methylene Disalicylate; Bacitracin Zinc;  
Bambermycins; Benzoylpas Calcium; Berythromycin ; Betamicin Sulfate; Biapenem;  
Biniramycin; Biphenamine Hydrochloride ; Bispyritione Magsulfex ; Butikacin;  
Butirosin Sulfate; Capreomycin Sulfate; Carbadox; Carbenicillin Disodium;
- 5 Carbenicillin Indanyl Sodium; Carbenicillin Phenyl Sodium; Carbenicillin Potassium;  
Carumonam Sodium; Cefaclor; Cefadroxil; Cefamandole; Cefamandole Nafate;  
Cefamandole Sodium; Cefaparole; Cefatrizine; Cefazaflur Sodium; Cefazolin; Cefazolin  
Sodium; Cefbuperazone; Cefdinir; Cefepime; Cefepime Hydrochloride; Cefetecol;  
Cefixime; Cefmenoxime Hydrochloride; Cefmetazole; Cefmetazole Sodium; Cefonicid
- 10 Monosodium; Cefonicid Sodium; Cefoperazone Sodium; Ceforanide; Cefotaxime  
Sodium; Cefotetan; Cefotetan Disodium; Cefotiam Hydrochloride; Cefoxitin; Cefoxitin  
Sodium; Cefpimizole; Cefpimizole Sodium; Cefpiramide; Cefpiramide Sodium;  
Cefpirome Sulfate; Cefpodoxime Proxetil; Cefprozil; Cefroxadine; Cefsulodin Sodium;  
Ceftazidime; Ceftibuten; Ceftizoxime Sodium; Ceftriaxone Sodium; Cefuroxime;
- 15 Cefuroxime Axetil; Cefuroxime Pivoxetil; Cefuroxime Sodium; Cephacetrile Sodium;  
Cephalexin; Cephalexin Hydrochloride; Cephaloglycin; Cephaloridine; Cephalothin  
Sodium; Cephapirin Sodium; Cephradine; Cetacycline Hydrochloride; Cetophenicol;  
Chloramphenicol ; Chloramphenicol Palmitate ; Chloramphenicol Pantothenate Complex  
; Chloramphenicol Sodium Succinate; Chlorhexidine Phosphanilate; Chloroxylenol;
- 20 Chlortetracycline Bisulfate ; Chlortetracycline Hydrochloride ; Cinoxacin;  
Ciprofloxacin; Ciprofloxacin Hydrochloride; Cirolemycin ; Clarithromycin;  
Clinafloxacin Hydrochloride; Clindamycin; Clindamycin Hydrochloride; Clindamycin  
Palmitate Hydrochloride; Clindamycin Phosphate; Clofazimine ; Cloxacillin Benzathine;  
Cloxacillin Sodium; Cloxyquin; Colistimethate Sodium; Colistin Sulfate; Coumermycin;
- 25 Coumermycin Sodium; Cyclacillin; Cycloserine; Dalfopristin; Dapsone ; Daptomycin;  
Demeclocycline; Demeclocycline Hydrochloride; Demecycline; Denofungin ;  
Diaveridine; Dicloxacillin; Dicloxacillin Sodium; Dihydrostreptomycin Sulfate;  
Dipyritione; Dirithromycin; Doxycycline; Doxycycline Calcium ; Doxycycline  
Fosfatex; Doxycycline Hyclate; Droxacin Sodium; Enoxacin; Epicillin; Epitetracycline
- 30 Hydrochloride; Erythromycin; Erythromycin Acistrate; Erythromycin Estolate;  
Erythromycin Ethylsuccinate; Erythromycin Gluceptate; Erythromycin Lactobionate;  
Erythromycin Propionate; Erythromycin Stearate; Ethambutol Hydrochloride;

- Ethionamide; Fleroxacin; Floxacillin; Fludalanine; Flumequine; Fosfomycin;  
Fosfomycin Tromethamine; Fumoxicillin; Furazolium Chloride; Furazolium Tartrate;  
Fusidate Sodium; Fusidic Acid; Gentamicin Sulfate; Gloximonam; Gramicidin;  
Haloprogin; Hetacillin; Hetacillin Potassium; Hexidine; Ibaflroxacin; Imipenem;  
5 Isoconazole; Isepamicin; Isoniazid; Josamycin; Kanamycin Sulfate; Kitasamycin;  
Levofuraltadone; Levopropylcillin Potassium; Lexithromycin; Lincomycin; Lincomycin  
Hydrochloride; Lomefloxacin; Lomefloxacin Hydrochloride; Lomefloxacin Mesylate;  
Loracarbef; Mafenide; Meclocycline; Meclocycline Sulfosalicylate; Megalomicin  
Potassium Phosphate; Mequidox; Meropenem; Methacycline; Methacycline  
10 Hydrochloride; Methenamine; Methenamine Hippurate; Methenamine Mandelate;  
Methicillin Sodium; Metioprim; Metronidazole Hydrochloride; Metronidazole  
Phosphate; Mezlocillin; Mezlocillin Sodium; Minocycline; Minocycline Hydrochloride;  
Mirincamycin Hydrochloride ; Monensin ; Monensin Sodium ; Nafcillin Sodium;  
Nalidixate Sodium; Nalidixic Acid; Natamycin; Nebramycin; Neomycin Palmitate;  
15 Neomycin Sulfate; Neomycin Undecylenate ; Netilmicin Sulfate; Neutramycin;  
Nifuradene; Nifuraldezone; Nifuratel ; Nifuratrone; Nifurdazil; Nifurimide; Nifurpirinol;  
Nifurquinazol; Nifurthiazole; Nitrocycline; Nitrofurantoin; Nitromide; Norfloxacin;  
Novobiocin Sodium; Ofloxacin; Ormetoprim; Oxacillin Sodium; Oximonam; Oximonam  
Sodium; Oxolinic Acid; Oxytetracycline; Oxytetracycline Calcium; Oxytetracycline  
20 Hydrochloride; Paldimycin; Parachlorophenol; Paulomycin; Pefloxacin; Pefloxacin  
Mesylate; Penamecillin; Penicillin G Benzathine; Penicillin G Potassium; Penicillin G  
Procaine; Penicillin G Sodium; Penicillin V; Penicillin V Benzathine; Penicillin V  
Hydrabamine; Penicillin V Potassium; Pentizidone Sodium; Phenyl Aminosalicylate;  
Piperacillin Sodium; Pirbenicillin Sodium; Piridicillin Sodium; Pirlimycin  
25 Hydrochloride; Pivampicillin Hydrochloride; Pivampicillin Pamoate; Pivampicillin  
Probenate; Polymyxin B Sulfate; Porfiromycin ; Propikacin; Pyrazinamide; Pyrithione  
Zinc; Quindecamine Acetate; Quinupristin; Racephenicol; Ramoplanin; Ranimycin;  
Relomycin; Repromycin; Rifabutin; Rifametane; Rifamexil; Rifamide; Rifampin;  
Rifapentine; Rifaximin; Rolitetracycline; Rolitetracycline Nitrate; Rosaramicin;  
30 Rosaramicin Butyrate; Rosaramicin Propionate; Rosaramicin Sodium Phosphate;  
Rosaramicin Stearate; Rosoxacin; Roxarsone; Roxithromycin; Sancycline; Sanfetrinem  
Sodium; Sarmoxicillin; Sarpicillin; Scopafungin ; Sisomicin; Sisomicin Sulfate;

- Sparfloxacin; Spectinomycin Hydrochloride; Spiramycin; Stallimycin Hydrochloride;  
Steffimycin; Streptomycin Sulfate; Streptonicozid; Sulfabenz ; Sulfabenzamide;  
Sulfacetamide; Sulfacetamide Sodium; Sulfcyline; Sulfadiazine; Sulfadiazine Sodium;  
Sulfadoxine; Sulfalene; Sulfamerazine; Sulfameter; Sulfamethazine; Sulfamethizole;  
5 Sulfamethoxazole; Sulfamonomethoxine; Sulfamoxole; Sulfanilate Zinc; Sulfantran ;  
Sulfasalazine; Sulfasomizole; Sulfathiazole; Sulfazamet; Sulfisoxazole; Sulfisoxazole  
Acetyl; Sulfisoxazole Diolamine; Sulfomyxin; Sulopenem; Sultamicillin; Suncillin  
Sodium; Talampicillin Hydrochloride; Teicoplanin; Temafloxacin Hydrochloride;  
Temocillin; Tetracycline; Tetracycline Hydrochloride; Tetracycline Phosphate Complex;
- 10 Tetroxoprim; Thiamphenicol; Thiphencillin Potassium; Ticarcillin Cresyl Sodium;  
Ticarcillin Disodium; Ticarcillin Monosodium; Ticlatone; Tiodonium Chloride;  
Tobramycin; Tobramycin Sulfate; Tosufloxacin; Trimethoprim; Trimethoprim Sulfate;  
Trisulfapyrimidines; Troleandomycin; Trospectomycin Sulfate; Tyrothricin;  
Vancomycin; Vancomycin Hydrochloride; Virginiamycin; and Zorbamycin.
- 15 Antiviral agents are compounds which prevent infection of cells by viruses or  
replication of the virus within the cell. There are many fewer antiviral drugs than  
antibacterial drugs because the process of viral replication is so closely related to DNA  
replication within the host cell, that non-specific antiviral agents would often be toxic to  
the host. There are several stages within the process of viral infection which can be  
20 blocked or inhibited by antiviral agents. These stages include, attachment of the virus to  
the host cell (immunoglobulin or binding peptides), uncoating of the virus (e.g.  
amantadine), synthesis or translation of viral mRNA (e.g. interferon), replication of viral  
RNA or DNA (e.g. nucleoside analogues), maturation of new virus proteins (e.g.  
protease inhibitors), and budding and release of the virus.
- 25 Nucleotide analogues are synthetic compounds which are similar to nucleotides,  
but which have an incomplete or abnormal deoxyribose or ribose group. Once the  
nucleotide analogues are in the cell, they are phosphorylated, producing the triphosphate  
formed which competes with normal nucleotides for incorporation into the viral DNA or  
RNA. Once the triphosphate form of the nucleotide analogue is incorporated into the  
30 growing nucleic acid chain, it causes irreversible association with the viral polymerase  
and thus chain termination. Nucleotide analogues include, but are not limited to,  
acyclovir (used for the treatment of herpes simplex virus and varicella-zoster virus),

gancyclovir (useful for the treatment of cytomegalovirus), idoxuridine, ribavirin (useful for the treatment of respiratory syncitial virus), dideoxyinosine, dideoxycytidine, and zidovudine (azidothymidine).

The interferons are cytokines which are secreted by virus-infected cells as well as immune cells. The interferons function by binding to specific receptors on cells adjacent to the infected cells, causing the change in the cell which protects it from infection by the virus.  $\alpha$  and  $\beta$ -interferon also induce the expression of Class I and Class II MHC molecules on the surface of infected cells, resulting in increased antigen presentation for host immune cell recognition.  $\alpha$  and  $\beta$ -interferons are available as recombinant forms and have been used for the treatment of chronic hepatitis B and C infection. At the dosages which are effective for anti-viral therapy, interferons have severe side effects such as fever, malaise and weight loss.

Immunoglobulin therapy is used for the prevention of viral infection.

Immunoglobulin therapy for viral infections is different than bacterial infections, because rather than being antigen-specific, the immunoglobulin therapy functions by binding to extracellular virions and preventing them from attaching to and entering cells which are susceptible to the viral infection. The therapy is useful for the prevention of viral infection for the period of time that the antibodies are present in the host. In general there are two types of immunoglobulin therapies, normal immunoglobulin therapy and hyper-immunoglobulin therapy. Normal immune globulin therapy utilizes a antibody product which is prepared from the serum of normal blood donors and pooled. This pooled product contains low titers of antibody to a wide range of human viruses, such as hepatitis A, parvovirus, enterovirus (especially in neonates). Hyper-immune globulin therapy utilizes antibodies which are prepared from the serum of individuals who have high titers of an antibody to a particular virus. Those antibodies are then used against a specific virus. Examples of hyper-immune globulins include zoster immune globulin (useful for the prevention of varicella in immuno-compromised children and neonates), human rabies immunoglobulin (useful in the post-exposure prophylaxis of a subject bitten by a rabid animal), hepatitis B immune globulin (useful in the prevention of hepatitis B virus, especially in a subject exposed to the virus), and RSV immune globulin (useful in the treatment of respiratory syncitial virus infections).

Another type of immunoglobulin therapy is active immunization. This involves the administration of antibodies or antibody fragments to viral surface proteins. Two types of vaccines which are available for active immunization of hepatitis B include serum-derived hepatitis B antibodies and recombinant hepatitis B antibodies. Both are prepared from HBsAg. The antibodies are administered in three doses to subjects at high risk of infection with hepatitis B virus, such as health care workers, sexual partners of chronic carriers, and infants.

- Thus, anti-viral agents useful in the invention include but are not limited to immunoglobulins, amantadine, interferon, nucleoside analogues, and protease inhibitors.
- Specific examples of anti-virals include but are not limited to Acemannan; Acyclovir; Acyclovir Sodium; Adefovir; Alovudine; Alvircept Sudotox; Amantadine Hydrochloride; Aranotin; Arildone; Atevirdine Mesylate; Avridine; Cidofovir; Cipamfylline; Cytarabine Hydrochloride; Delavirdine Mesylate; Desciclovir; Didanosine; Disoxaril; Edoxudine; Enviradene; Enviroxime; Famciclovir; Famotine Hydrochloride; Fiacitabine; Fialuridine; Fosarilate; Foscarnet Sodium; Fosfonet Sodium; Ganciclovir; Ganciclovir Sodium; Idoxuridine; Kethoxal; Lamivudine; Lobucavir; Memantine Hydrochloride; Methisazone; Nevirapine; Penciclovir; Pirodavir; Ribavirin; Rimantadine Hydrochloride; Saquinavir Mesylate; Somantadine Hydrochloride; Sorivudine; Statolon; Stavudine; Tilorone Hydrochloride; Trifluridine; Valacyclovir Hydrochloride; Vidarabine; Vidarabine Phosphate; Vidarabine Sodium Phosphate; Viroxime; Zalcitabine; Zidovudine; and Zinviroxime.

Anti-fungal agents are useful for the treatment and prevention of infective fungi. Anti-fungal agents are sometimes classified by their mechanism of action. Some anti-fungal agents function as cell wall inhibitors by inhibiting glucose synthase. These include, but are not limited to, basiungin/ECB. Other anti-fungal agents function by destabilizing membrane integrity. These include, but are not limited to, imidazoles, such as clotrimazole, sertaconazole, fluconazole, itraconazole, ketoconazole, miconazole, and voriconazole, as well as FK 463, amphotericin B, BAY 38-9502, MK 991, pradimicin, UK 292, butenafine, and terbinafine. Other anti-fungal agents function by breaking down chitin (e.g. chitinase) or immunosuppression (S01 cream). Some examples of commercially-available agents are shown in Table B

Table B

| Company            | Brand Name            | Generic Name              | Indication                                | Mechanism of Action   |
|--------------------|-----------------------|---------------------------|---|---|
| PHARMACIA & UPJOHN | PNU 196443            | PNU 196443                | Anti Fungal                               | n/k   |
| Lily               | LY 303366             | Basisungn/ECB             | Fungal Infections                         | Anti-fungal/cell wall inhibitor, glucose synthase inhibitor |
| Bayer              | Canesten              | Clofotrimazole            | Fungal Infections                         | Membrane integrity destabilizer                             |
| Fujiisawa          | FK 463                | FK 463                    | Fungal Infections                         | Membrane integrity destabilizer                             |
| Mylan              | Sertaconazole         | Sertaconazole             | Fungal Infections                         | Membrane integrity destabilizer                             |
| Genzyme            | Chitinase             | Chitinase                 | Fungal Infections, Systemic               | Chitin Breakdown  |
| Liposome           | Abelcet               | Amphotericin B, Liposomal | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Sequus             | Amphotec              | Amphotericin B, Liposomal | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Bayer              | BAY 38-9502           | BAY 38-9502               | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Pfizer             | Diflucan              | Fluconazole               | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Johnson & Johnson  | Sporanox              | Itraconazole              | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Sepracor           | Itraconazole (2R, 4S) | Itraconazole (2R, 4S)     | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Johnson & Johnson  | Nizoral               | Ketoconazole              | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Johnson & Johnson  | Monistat              | Miconazole                | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Merck              | MK 991                | MK 991                    | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Bristol Myers Sq'b | Pradimicin            | Pradimicin                | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Pfizer             | UK-292, 663           | UK-292, 663               | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Pfizer             | Voriconazole          | Voriconazole              | Fungal Infections, Systemic               | Membrane integrity destabilizer                             |
| Mylan              | 501 Cream             | 501 Cream                 | Inflammatory Fungal Conditions            | Immunosuppression   |
| Mylan              | Mentax                | Butenafine                | Nail Fungus                               | Membrane Integrity Destabiliser                             |
| Schering Plough    | Anti Fungal           | Anti Fungal               | Opportunistic Infections                  | Membrane Integrity Destabiliser                             |
| Alza               | Mycetex Troche        | Clofotrimazole            | Oral Thrush                               | Membrane Integrity Stabiliser                               |
| Novartis           | Lamisil               | Terbinafine               | Systemic Fungal Infections, Onychomycosis | Membrane Integrity Destabiliser                             |

Thus, the anti-fungal agents useful in the invention include but are not limited to

- imidazoles, FK 463, amphotericin B, BAY 38-9502, MK 991, pradimicin, UK 292, butenafine, chitinase, 501 cream, Acrisorcin; Ambruticin; Amorolfine, Amphotericin B; Azaconazole; Azaserine; Basifungin; Bifonazole; Biphenamine Hydrochloride ; 5 Bispyrithione Magsulfex ; Butoconazole Nitrate; Calcium Undecylenate; Candicidin; Carbol-Fuchs; Chlordantoin; Ciclopirox; Ciclopirox Olamine; Cilofungin; Cisconazole; Clotrimazole; Cuprimyxin ; Denofungin ; Dipyrithione; Doconazole; Econazole; Econazole Nitrate; Enilconazole; Ethonam Nitrate; Fenticonazole Nitrate; Filipin; Fluconazole; Flucytosine; Fungimycin; Griseofulvin; Hamycin; Isoconazole ; 10 Itraconazole; Kalafungin; Ketoconazole; Lomofungin; Lydimycin; Meparticin ; Miconazole; Miconazole Nitrate; Monensin ; Monensin Sodium ; Naftifine Hydrochloride; Neomycin Undecylenate ; Nifuratel ; Nifurmerone; Nitralamine Hydrochloride; Nystatin; Octanoic Acid; Orconazole Nitrate; Oxiconazole Nitrate; Oxifungin Hydrochloride; Parconazole Hydrochloride; Partricin ; Potassium Iodide ; 15 Proclanol ; Pyritohone Zinc ; Pyrrolnitrin; Rutamycin; Sanguinarium Chloride ; Saperconazole; Scopafungin ; Selenium Sulfide ; Sinefungin; Sulconazole Nitrate; Terbinafine; Terconazole; Thiram; Ticlatone ; Tioconazole; Tolciclate; Tolindate;

Tolnaftate; Triacetin; Triafungin; Undecylenic Acid; Viridofulvin; Zinc Undecylenate; and Zinoconazole Hydrochloride.

Immunostimulatory nucleic acids can be combined with other therapeutic agents such as adjuvants to enhance immune responses. The immunostimulatory nucleic acid and other therapeutic agent may be administered simultaneously or sequentially. When the other therapeutic agents are administered simultaneously they can be administered in the same or separate formulations, but are administered at the same time. The other therapeutic agents are administered sequentially with one another and with immunostimulatory nucleic acid, when the administration of the other therapeutic agents and the immunostimulatory nucleic acid is temporally separated. The separation in time between the administration of these compounds may be a matter of minutes or it may be longer. Other therapeutic agents include but are not limited to adjuvants, cytokines, antibodies, antigens, etc.

The immunostimulatory nucleic acids are useful as adjuvants for inducing a systemic immune response. Thus either can be delivered to a subject exposed to an antigen to produce an enhanced immune response to the antigen.

In addition to the immunostimulatory nucleic acids, the compositions of the invention may also be administered with non-nucleic acid adjuvants. A non-nucleic acid adjuvant is any molecule or compound except for the immunostimulatory nucleic acids described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that create a depo effect, immune stimulating adjuvants, and adjuvants that create a depo effect and stimulate the immune system.

An adjuvant that creates a depo effect as used herein is an adjuvant that causes the antigen to be slowly released in the body, thus prolonging the exposure of immune cells to the antigen. This class of adjuvants includes but is not limited to alum (e.g., aluminum hydroxide, aluminum phosphate); or emulsion-based formulations including mineral oil, non-mineral oil, water-in-oil or oil-in-water-in oil emulsion, oil-in-water emulsions such as Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720, AirLiquide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, CA; and PROVAX (an oil-in-water

emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC, Pharmaceuticals Corporation, San Diego, CA).

- An immune stimulating adjuvant is an adjuvant that causes activation of a cell of the immune system. It may, for instance, cause an immune cell to produce and secrete cytokines. This class of adjuvants includes but is not limited to saponins purified from the bark of the *Q. saponaria* tree, such as QS21 (a glycolipid that elutes in the 21<sup>st</sup> peak with HPLC fractionation; Aquila Biopharmaceuticals, Inc., Worcester, MA); poly[di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA); derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, MT), muramyl dipeptide (MDP; Ribi) and threonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland); and Leishmania elongation factor (a purified *Leishmania* protein; Corixa Corporation, Seattle, WA).

Adjuvants that create a depo effect and stimulate the immune system are those compounds which have both of the above- identified functions. This class of adjuvants includes but is not limited to ISCOMS (Immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia); SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21; SmithKline Beecham Biologicals [SBB], Rixensart, Belgium); SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium); non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxpropylene flanked by chains of polyoxyethylene; Vaxcel, Inc., Norcross, GA); and Syntex Adjuvant Formulation (SAF, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, CO).

The immunostimulatory nucleic acids are also useful as mucosal adjuvants. It has previously been discovered that both systemic and mucosal immunity are induced by mucosal delivery of CpG nucleic acids. The systemic immunity induced in response to CpG nucleic acids included both humoral and cell-mediated responses to specific antigens that were not capable of inducing systemic immunity when administered alone to the mucosa. Furthermore, both CpG nucleic acids and cholera toxin (CT, a mucosal adjuvant that induces a Th2-like response) induced CTL. This was surprising since with

systemic immunization, the presence of Th2-like antibodies is normally associated with a lack of CTL (Schirrmbeck *et al.*, 1995). Based on the results presented herein it is expected that the immunostimulatory nucleic acids will function in a similar manner.

- Additionally, the immunostimulatory nucleic acids induce a mucosal response at both local (e.g., lung) and remote (e.g., lower digestive tract) mucosal sites. Significant levels of IgA antibodies are induced at distant mucosal sites by the immunostimulatory nucleic acids. CT is generally considered to be a highly effective mucosal adjuvant. As has been previously reported (Snider 1995), CT induces predominantly IgG1 isotype of antibodies, which are indicative of Th2-type response. In contrast, the immunostimulatory nucleic acids are more Th1 with predominantly IgG2a antibodies, especially after boost or when the two adjuvants are combined. Th1-type antibodies in general have better neutralizing capabilities, and furthermore, a Th2 response in the lung is highly undesirable because it is associated with asthma (Kay, 1996, Hogg, 1997). Thus the use of immunostimulatory nucleic acids as a mucosal adjuvant has benefits that other mucosal adjuvants cannot achieve. The immunostimulatory nucleic acids of the invention also are useful as mucosal adjuvants for induction of both a systemic and a mucosal immune response.

- Mucosal adjuvants referred to as non-nucleic acid mucosal adjuvants may also be administered with the Immunostimulatory nucleic acids. A non-nucleic acid mucosal adjuvant as used herein is an adjuvant other than a immunostimulatory nucleic acid that is capable of inducing a mucosal immune response in a subject when administered to a mucosal surface in conjunction with an antigen. Mucosal adjuvants include but are not limited to Bacterial toxins e.g., Cholera toxin (CT), CT derivatives including but not limited to CT B subunit (CTB) (Wu *et al.*, 1998, Tochikubo *et al.*, 1998); CTD53 (Val to Asp) (Fontana *et al.*, 1995); CTK97 (Val to Lys) (Fontana *et al.*, 1995); CTK104 (Tyr to Lys) (Fontana *et al.*, 1995); CTD53/K63 (Val to Asp, Ser to Lys) (Fontana *et al.*, 1995); CTH54 (Arg to His) (Fontana *et al.*, 1995); CTN107 (His to Asn) (Fontana *et al.*, 1995); CTE114 (Ser to Glu) (Fontana *et al.*, 1995); CTE112K (Glu to Lys) (Yamamoto *et al.*, 1997a); CTS61F (Ser to Phe) (Yamamoto *et al.*, 1997a, 1997b); CTS106 (Pro to Lys) (Douce *et al.*, 1997, Fontana *et al.*, 1995); and CTK63 (Ser to Lys) (Douce *et al.*, 1997, Fontana *et al.*, 1995), Zonula occludens toxin, zot, Escherichia coli heat-labile enterotoxin, Labile Toxin (LT), LT derivatives including but not limited to LT B subunit

(LTB) (Verweij et al., 1998); LT7K (Arg to Lys) (Komase et al., 1998, Douce et al., 1995); LT61F (Ser to Phe) (Komase et al., 1998); LT112K (Glu to Lys) (Komase et al., 1998); LT118E (Gly to Glu) (Komase et al., 1998); LT146E (Arg to Glu) (Komase et al., 1998); LT192G (Arg to Gly) (Komase et al., 1998); LTK63 (Ser to Lys) (Marchetti et al., 1998, Douce et al., 1997, 1998, Di Tommaso et al., 1996); and LTR72 (Ala to Arg) (Giuliani et al., 1998), Pertussis toxin, PT. (Lycke et al., 1992, Spangler BD, 1992, Freytag and Clemments, 1999, Roberts et al., 1995, Wilson et al., 1995) including PT-9K/129G (Roberts et al., 1995, Cropley et al., 1995); Toxin derivatives (see below) (Holmgren et al., 1993, Verweij et al., 1998, Rappuoli et al., 1995, Freytag and Clemments, 1999); Lipid A derivatives (e.g., monophosphoryl lipid A, MPL) (Sasaki et al., 1998, Vancott et al., 1998; Muramyl Dipeptide (MDP) derivatives (Fukushima et al., 1996, Ogawa et al., 1989, Michalek et al., 1983, Morisaki et al., 1983); Bacterial outer membrane proteins (e.g., outer surface protein A (OspA) lipoprotein of *Borrelia burgdorferi*, outer membrane protine of *Neisseria meningitidis*) (Marinaro et al., 1999, Van de Verg et al., 1996); Oil-in-water emulsions (e.g., MF59) (Barchfield et al., 1999, Verschoor et al., 1999, O'Hagan, 1998); Aluminum salts (Isaka et al., 1998, 1999); and Saponins (e.g., QS21) Aquila Biopharmaceuticals, Inc., Worster, MA) (Sasaki et al., 1998, MacNeal et al., 1998), ISCOMS, MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, CA); the Seppic ISA series of Montanide adjuvants (e.g., Montanide ISA 720; AirLiquide, Paris, France); PROVAX (an oil-in-water emulsion containing a stabilizing detergent and a micelle-forming agent; IDEC Pharmaceuticals Corporation, San Diego, CA); Syntex Adjuvant Formulation (SAF; Syntex Chemicals, Inc., Boulder, CO); poly[di(carboxylatophenoxy)phosphazene (PCPP polymer, Virus Research Institute, USA) and Leishmania elongation factor (Corixa Corporation, Seattle, WA).

Immune responses can also be induced or augmented by the co-administration or co-linear expression of cytokines (Bueler & Mulligan, 1996; Chow et al., 1997; Geissler et al., 1997; Iwasaki et al., 1997; Kim et al., 1997) or B-7 co-stimulatory molecules (Iwasaki et al., 1997; Tsuji et al., 1997) with the Immunostimulatory nucleic acids. The cytokines can be administered directly with Immunostimulatory nucleic acids or may be administered in the form of a nucleic acid vector that encodes the cytokine, such that the cytokine can be expressed *in vivo*. In one embodiment, the cytokine is administered in

- the form of a plasmid expression vector. The term cytokine is used as a generic name for a diverse group of soluble proteins and peptides which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also
- 5 mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Examples of cytokines include, but are not limited to IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-15, IL-18, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interferon- $\gamma$  ( $\gamma$ -IFN), IFN- $\alpha$ , tumor necrosis factor (TNF), TGF- $\beta$ , FLT-3 ligand, and CD40 ligand.
- 10 Cytokines play a role in directing the T cell response. Helper (CD4+) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immune system cells, including other T cells. Most mature CD4+ T helper cells express one of two cytokine profiles: Th1 or Th2. The Th1 subset promotes delayed-type hypersensitivity, cell-mediated immunity, and immunoglobulin class
- 15 switching to IgG<sub>2a</sub>. The Th2 subset induces humoral immunity by activating B cells, promoting antibody production, and inducing class switching to IgG<sub>1</sub> and IgE. In some embodiments, it is preferred that the cytokine be a Th1 cytokine.

The nucleic acids are also useful for redirecting an immune response from a Th2 immune response to a Th1 immune response. Redirection of an immune response from a

20 Th2 to a Th1 immune response can be assessed by measuring the levels of cytokines produced in response to the nucleic acid (e.g., by inducing monocytic cells and other cells to produce Th1 cytokines, including IL-12, IFN- $\gamma$  and GM-CSF). The redirection or rebalance of the immune response from a Th2 to a Th1 response is particularly useful for the treatment or prevention of asthma. For instance, an effective amount for treating

25 asthma can be that amount; useful for redirecting a Th2 type of immune response that is associated with asthma to a Th1 type of response. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotype switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially

30 IFN- $\gamma$  and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. The immunostimulatory nucleic acids of the invention cause an increase in

Th1 cytokines which helps to rebalance the immune system, preventing or reducing the adverse effects associated with a predominately Th2 immune response.

- The nucleic acids are also useful for improving survival, differentiation, activation and maturation of dendritic cells. The immunostimulatory nucleic acids have
- 5 the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells. Dendritic precursor cells isolated from blood by immunomagnetic cell sorting develop morphologic and functional characteristics of dendritic cells during a two day incubation with GM-CSF. Without GM-CSF these cells undergo apoptosis. The immunostimulatory nucleic acids are superior to GM-CSF in promoting survival and
- 10 differentiation of dendritic cells (MHC II expression, cell size, granularity). The immunostimulatory nucleic acids also induce maturation of dendritic cells. Since dendritic cells form the link between the innate and the acquired immune system, by presenting antigens as well as through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local environment, the ability to
- 15 activate dendritic cells with immunostimulatory nucleic acids supports the use of these immunostimulatory nucleic acid based strategies for *in vivo* and *ex-vivo* immunotherapy against disorders such as cancer and allergic or infectious diseases. The immunostimulatory nucleic acids are also useful for activating and inducing maturation of dendritic cells.
- 20 Immunostimulatory nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). ADCC can be performed using a immunostimulatory nucleic acid in combination with an antibody specific for a cellular target, such as a cancer cell. When the immunostimulatory nucleic acid is administered to a subject in conjunction with the antibody the subject's immune system is induced to
- 25 kill the tumor cell. The antibodies useful in the ADCC procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available. Examples of these antibodies are listed below among the list of cancer immunotherapies.
- The immunostimulatory nucleic acids may also be administered in conjunction
- 30 with an anti-cancer therapy. Anti-cancer therapies include cancer medicaments, radiation and surgical procedures. As used herein, a "cancer medicament" refers to a agent which is administered to a subject for the purpose of treating a cancer. As used

herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medicament is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of  
5 medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

As used herein, a "cancer medicament" refers to an agent which is administered  
10 to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medicament is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medicaments for the  
15 treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers. Additionally, the methods of the invention are intended to embrace the use of more than one cancer medicament along with the immunostimulatory nucleic acids. As an example, where  
20 appropriate, the immunostimulatory nucleic acids may be administered with both a chemotherapeutic agent and an immunotherapeutic agent. Alternatively, the cancer medicament may embrace an immunotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine all administered to one subject for the  
25 purpose of treating a subject having a cancer or at risk of developing a cancer.

Cancer medicaments function in a variety of ways. Some cancer medicaments work by targeting physiological mechanisms that are specific to tumor cells. Examples include the targeting of specific genes and their gene products (i.e., proteins primarily) which are mutated in cancers. Such genes include but are not limited to oncogenes (e.g.,  
30 Ras, Her2, bcl-2), tumor suppressor genes (e.g., EGF, p53, Rb), and cell cycle targets (e.g., CDK4, p21, telomerase). Cancer medicaments can alternately target signal transduction pathways and molecular mechanisms which are altered in cancer cells.

Targeting of cancer cells via the epitopes expressed on their cell surface is accomplished through the use of monoclonal antibodies. This latter type of cancer medicament is generally referred to herein as immunotherapy.

- Other cancer medicaments target cells other than cancer cells. For example,
- 5 some medicaments prime the immune system to attack tumor cells (i.e., cancer vaccines). Still other medicaments, called angiogenesis inhibitors, function by attacking the blood supply of solid tumors. Since the most malignant cancers are able to metastasize (i.e., exist the primary tumor site and seed a distal tissue, thereby forming a secondary tumor), medicaments that impede this metastasis are also useful in the treatment of cancer. Angiogenic mediators include basic FGF, VEGF, angiopoietins, angiostatin, endostatin, TNF $\alpha$ , TNP-470, thrombospondin-1, platelet factor 4, CAI, and certain members of the integrin family of proteins. One category of this type of medicament is a metalloproteinase inhibitor, which inhibits the enzymes used by the cancer cells to exit the primary tumor site and extravasate into another tissue.
  - 10
  - 15 Some cancer cells are antigenic and thus can be targeted by the immune system. In one aspect, the combined administration of immunostimulatory nucleic acids and cancer medicaments, particularly those which are classified as cancer immunotherapies, is useful for stimulating a specific immune response against a cancer antigen. A "cancer antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens, such as those present in cancer vaccines or those used to prepare cancer immunotherapies, can be prepared from crude cancer cell extracts, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, or by partially purifying the antigens, using recombinant technology, or de novo synthesis of known antigens.
  - 20
  - 25 Cancer antigens can be used in the form of immunogenic portions of a particular antigen or in some instances a whole cell or a tumor mass can be used as the antigen. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.
  - 30 The theory of immune surveillance is that a prime function of the immune system is to detect and eliminate neoplastic cells before a tumor forms. A basic principle of this theory is that cancer cells are antigenically different from normal cells and thus elicit

immune reactions that are similar to those that cause rejection of immunologically incompatible allografts. Studies have confirmed that tumor cells differ, either qualitatively or quantitatively, in their expression of antigens. For example, "tumor-specific antigens" are antigens that are specifically associated with tumor cells but not normal cells. Examples of tumor specific antigens are viral antigens in tumors induced by DNA or RNA viruses. "Tumor-associated" antigens are present in both tumor cells and normal cells but are present in a different quantity or a different form in tumor cells.

5 Examples of such antigens are oncofetal antigens (e.g., carcinoembryonic antigen), differentiation antigens (e.g., T and Tn antigens), and oncogene products (e.g., HER/neu).

10

Different types of cells that can kill tumor targets *in vitro* and *in vivo* have been identified: natural killer cells (NK cells), cytolytic T lymphocytes (CTLs), lymphokine-activated killer cells (LAKs), and activated macrophages. NK cells can kill tumor cells without having been previously sensitized to specific antigens, and the activity does not require the presence of class I antigens encoded by the major histocompatibility complex (MHC) on target cells. NK cells are thought to participate in the control of nascent tumors and in the control of metastatic growth. In contrast to NK cells, CTLs can kill tumor cells only after they have been sensitized to tumor antigens and when the target antigen is expressed on the tumor cells that also express MHC class I. CTLs are thought to be effector cells in the rejection of transplanted tumors and of tumors caused by DNA viruses. LAK cells are a subset of null lymphocytes distinct from the NK and CTL populations. Activated macrophages can kill tumor cells in a manner that is not antigen dependent nor MHC restricted once activated. Activated macrophages are thought to decrease the growth rate of the tumors they infiltrate. *In vitro* assays have identified other immune mechanisms such as antibody-dependent, cell-mediated cytotoxic reactions and lysis by antibody plus complement. However, these immune effector mechanisms are thought to be less important *in vivo* than the function of NK, CTLs, LAK, and macrophages *in vivo* (for review see Piessens, W.F., and David, J., "Tumor Immunology", In: Scientific American Medicine, Vol. 2, Scientific American Books, N.Y., pp. 1-13, 1996.

The goal of immunotherapy is to augment a patient's immune response to an established tumor. One method of immunotherapy includes the use of adjuvants.

Adjuvant substances derived from microorganisms, such as bacillus Calmette-Guerin, heighten the immune response and enhance resistance to tumors in animals.

Immunotherapeutic agents are medicaments which derive from antibodies or antibody fragments which specifically bind or recognize a cancer antigen. As used herein a cancer antigen is broadly defined as an antigen expressed by a cancer cell. Preferably, the antigen is expressed at the cell surface of the cancer cell. Even more preferably, the antigen is one which is not expressed by normal cells, or at least not expressed to the same level as in cancer cells. Antibody-based immunotherapies may function by binding to the cell surface of a cancer cell and thereby stimulate the endogenous immune system to attack the cancer cell. Another way in which antibody-based therapy functions is as a delivery system for the specific targeting of toxic substances to cancer cells. Antibodies are usually conjugated to toxins such as ricin (e.g., from castor beans), calicheamicin and maytansinoids, to radioactive isotopes such as Iodine-131 and Yttrium-90, to chemotherapeutic agents (as described herein), or to biological response modifiers. In this way, the toxic substances can be concentrated in the region of the cancer and non-specific toxicity to normal cells can be minimized. In addition to the use of antibodies which are specific for cancer antigens, antibodies which bind to vasculature, such as those which bind to endothelial cells, are also useful in the invention. This is because generally solid tumors are dependent upon newly formed blood vessels to survive, and thus most tumors are capable of recruiting and stimulating the growth of new blood vessels. As a result, one strategy of many cancer medicaments is to attack the blood vessels feeding a tumor and/or the connective tissues (or stroma) supporting such blood vessels.

The use of immunostimulatory nucleic acids in conjunction with immunotherapeutic agents such as monoclonal antibodies is able to increase long-term survival through a number of mechanisms including significant enhancement of ADCC (as discussed above), activation of natural killer (NK) cells and an increase in IFN $\alpha$  levels. The nucleic acids when used in combination with monoclonal antibodies serve to reduce the dose of the antibody required to achieve a biological result.

Examples of cancer immunotherapies which are currently being used or which are in development are listed in Table C.

Table C

| Cancer Immunotherapies in Development or on the Market   |  |   |
|--|--|---|
| MARKETER   | BRAND NAME (GENERIC NAME)  | INDICATION  |
| IDEC/Genentech, Inc./Hoffmann-LaRoche (first monoclonal antibody licensed for the treatment of cancer in the U.S.)           | Rituxan™ (rituximab, Mabthera) (IDEC-C2B8, chimeric murine/human anti-CD20 MAb ) | non-Hodgkin's lymphoma                            |
| Genentech/Hoffmann-La Roche  | Herceptin, anti-Her2 hMAb  | Breast/ovarian                                    |
| Cytogen Corp.  | Quadramet (CYT-424) radiotherapeutic agent                                       | Bone metastases                                   |
| Centocor/Glaxo/Ajinomoto   | Panorex® (17-1A) (murine monoclonal antibody)                                    | Adjuvant therapy for colorectal (Dukes-C)         |
| Centocor/Ajinomoto   | Panorex® (17-1A) (chimeric murine monoclonal antibody)                           | Pancreatic, lung, breast, ovary                   |
| IDEC   | IDEC-Y2B8 (murine, anti-CD20 MAb labeled with Yttrium-90)                        | non-Hodgkin's lymphoma                            |
| ImClone Systems  | BEC2 (anti-idiotypic MAb, mimics the GD <sub>3</sub> epitope) (with BCG)         | Small cell lung                                   |
| ImClone Systems  | C225 (chimeric monoclonal antibody to epidermal growth factor receptor (EGFr))   | Renal cell  |
| Technicclone International/Alpha Therapeutics  | Oncolym (Lym-1 monoclonal antibody linked to 131 iodine)                         | non-Hodgkin's lymphoma                            |
| Protein Design Labs  | SMART M195 Ab, humanized   | Acute myeloid leukemia                            |
| Technicclone Corporation/Cambridge Antibody Technology   | <sup>131</sup> I LYM-1 (Oncolym™)  | non-Hodgkin's lymphoma                            |
| Aronex Pharmaceuticals, Inc.   | ATRAGEN®   | Acute promyelocytic leukemia                      |
| ImClone Systems  | C225 (chimeric anti-EGFr monoclonal antibody) + cisplatin or radiation           | Head & neck, non-small cell lung cancer           |
| Altarex, Canada  | Ovarex (B43.13, anti-idiotypic CA 125, mouse MAb)                                | Ovarian   |
| Coulter Pharma (Clinical results have been positive, but the drug has been associated with significant bone marrow toxicity) | Bexxar (anti-CD20 Mab labeled with <sup>131</sup> I)                             | non-Hodgkin's lymphoma                            |
| Aronex Pharmaceuticals, Inc.   | ATRAGEN®   | Kaposi's sarcoma                                  |
| IDEC Pharmaceuticals Corp./Genentech   | Rituxan™ (MAb against CD20) pan-B Ab in combo. with chemotherapy                 | B cell lymphoma                                   |
| LeukoSite/Ilex Oncology  | LDP-03, huMAb to the leukocyte antigen CAMPATH                                   | Chronic lymphocytic leukemia (CLL)                |
| Center of Molecular Immunology   | ior t6 (anti CD6, murine MAb) CTCL   | Cancer  |
| Medarex/Novartis   | MDX-210 (humanized anti-HER-2 bispecific antibody)                               | Breast, ovarian                                   |
| Medarex/Novartis   | MDX-210 (humanized anti-HER-2 bispecific antibody)                               | Prostate, non-small cell lung, pancreatic, breast |

|                                     |  |  |
|-------------------------------------|--|--|
| Medarex                             | MDX-11 (complement activating receptor (CAR) monoclonal antibody)                      | Acute myelogenous leukemia (AML)   |
| Medarex/Novartis                    | MDX-210 (humanized anti-HER-2 bispecific antibody)                                     | Renal and colon  |
| Medarex                             | MDX-11 (complement activating receptor (CAR) monoclonal antibody)                      | Ex vivo bone marrow purging in acute myelogenous leukemia (AML)                |
| Medarex                             | MDX-22 (humanized bispecific antibody, MAb-conjugates) (complement cascade activators) | Acute myeloid leukemia   |
| Cytogen                             | OV103 (Yttrium-90 labelled antibody)   | Ovarian  |
| Cytogen                             | OV103 (Yttrium-90 labelled antibody)   | Prostate   |
| Aronex Pharmaceuticals, Inc.        | ATRAGEN®   | non-Hodgkin's lymphoma   |
| Glaxo Wellcome plc                  | 3622W94 MAb that binds to EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas        | non-small cell lung, prostate (adjuvant)                                       |
| Genentech                           | Anti-VEGF, RhuMAb (inhibits angiogenesis)  | Lung, breast, prostate, colorectal   |
| Protein Design Labs                 | Zenapax (SMART Anti-Tac (IL-2 receptor) Ab, humanized)                                 | Leukemia, lymphoma   |
| Protein Design Labs                 | SMART M195 Ab, humanized   | Acute promyelocytic leukemia   |
| ImClone Systems                     | C225 (chimeric anti-EGFr monoclonal antibody ) + taxol                                 | Breast   |
| ImClone Systems (licensed from RPR) | C225 (chimeric anti-EGFr monoclonal antibody ) + doxorubicin                           | prostate   |
| ImClone Systems                     | C225 (chimeric anti-EGFr monoclonal antibody ) + adriamycin                            | prostate   |
| ImClone Systems                     | BEC2 (anti-idiotypic MAb, mimics the GD <sub>3</sub> epitope)                          | Melanoma   |
| Medarex                             | MDX-210 (humanized anti-HER-2 bispecific antibody)                                     | Cancer   |
| Medarex                             | MDX-220 (bispecific for tumors that express TAG-72)                                    | Lung, colon, prostate, ovarian, endometrial, pancreatic and gastric            |
| Medarex/Novartis                    | MDX-210 (humanized anti-HER-2 bispecific antibody)                                     | Prostate   |
| Medarex/Merck KgaA                  | MDX-447 (humanized anti-EGF receptor bispecific antibody)                              | EGF receptor cancers (head & neck, prostate, lung, bladder, cervical, ovarian) |
| Medarex/Novartis                    | MDX-210 (humanized anti-HER-2 bispecific antibody)                                     | Comb. Therapy with G-CSF for various cancers, esp. breast                      |
| IDEC                                | MELIMMUNE-2 (murine monoclonal antibody therapeutic vaccine )                          | Melanoma   |
| IDEC                                | MELIMMUNE-1 (murine monoclonal antibody therapeutic vaccine )                          | Melanoma   |

|  |   |                                  |
|--|---|----------------------------------|
| Immunomedics, Inc.   | CEACIDE™ (I-131)  | Colorectal and other             |
| NeoRx  | Pretarget™ radioactive antibodies                           | non-Hodgkin's B cell lymphoma    |
| Novopharm Biotech, Inc.                                      | NovoMAb-G2 (pancarcinoma specific Ab)                       | Cancer                           |
| Technicclone Corporation/<br>Cambridge Antibody Technology   | TNT (chimeric MAb to histone antigens)                      | Brain                            |
| Technicclone International/<br>Cambridge Antibody Technology | TNT (chimeric MAb to histone antigens)                      | Brain                            |
| Novopharm  | Gliomab-H (Monoclonals - Humanized Abs)                     | Brain, melanomas, neuroblastomas |
| Genetics Institute/AHP                                       | GNI-250 Mab   | Colorectal                       |
| Merck KgaA   | EMD-72000 (chimeric-EGF antagonist)                         | Cancer                           |
| Immunomedics   | LymphoCide (humanized LL2 antibody)                         | non-Hodgkin's B-cell lymphoma    |
| Immunex/AHP  | CMA 676 (monoclonal antibody conjugate)                     | Acute myelogenous leukemia       |
| Novopharm Biotech, Inc.                                      | Monopharm-C   | Colon, lung, pancreatic          |
| Novopharm Biotech, Inc.                                      | 4B5 anti-idiotype Ab  | Melanoma, small-cell lung        |
| Center of Molecular Immunology                               | ior egf/r3 (anti EGF-R humanized Ab)                        | Radioimmunotherapy               |
| Center of Molecular Immunology                               | ior c5 (murine MAb colorectal) for radioimmunotherapy       | Colorectal                       |
| Creative BioMolecules/<br>Chiron                             | BABS (biosynthetic antibody binding site)<br>Proteins       | Breast cancer                    |
| ImClone Systems/Chugai                                       | FLK-2 (monoclonal antibody to fetal liver kinase-2 (FLK-2)) | Tumor-associated angiogenesis    |
| ImmunoGen, Inc.  | Humanized MAb/small-drug conjugate                          | Small-cell lung                  |
| Medarex, Inc.  | MDX-260 bispecific, targets GD-2                            | Melanoma, glioma, neuroblastoma  |
| Procyon Biopharma, Inc.                                      | ANA Ab  | Cancer                           |
| Protein Design Labs  | SMART ID10 Ab   | B-cell lymphoma                  |
| Protein Design Labs/Novartis                                 | SMART ABL 364 Ab  | Breast, lung, colon              |
| Immunomedics, Inc.   | ImmucRATT-CEA   | Colorectal                       |

Yet other types of chemotherapeutic agents which can be used according to the invention include Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorambucil, Cytarabine HCl, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Flouxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl

(nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o,p'-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-AMSA), Azacitidine, Erythropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; 5 methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate.

Cancer vaccines are medicaments which are intended to stimulate an endogenous immune response against cancer cells. Currently produced vaccines predominantly activate the humoral immune system (i.e., the antibody dependent immune response).

10 Other vaccines currently in development are focused on activating the cell-mediated immune system including cytotoxic T lymphocytes which are capable of killing tumor cells. Cancer vaccines generally enhance the presentation of cancer antigens to both antigen presenting cells (e.g., macrophages and dendritic cells) and/or to other immune cells such as T cells, B cells, and NK cells.

15 Although cancer vaccines may take one of several forms, as discussed infra, their purpose is to deliver cancer antigens and/or cancer associated antigens to antigen presenting cells (APC) in order to facilitate the endogenous processing of such antigens by APC and the ultimate presentation of antigen presentation on the cell surface in the context of MHC class I molecules. One form of cancer vaccine is a whole cell vaccine 20 which is a preparation of cancer cells which have been removed from a subject, treated ex vivo and then reintroduced as whole cells in the subject. Lysates of tumor cells can also be used as cancer vaccines to elicit an immune response. Another form cancer vaccine is a peptide vaccine which uses cancer-specific or cancer-associated small proteins to activate T cells. Cancer-associated proteins are proteins which are not 25 exclusively expressed by cancer cells (i.e., other normal cells may still express these antigens). However, the expression of cancer-associated antigens is generally consistently upregulated with cancers of a particular type. Yet another form of cancer vaccine is a dendritic cell vaccine which includes whole dendritic cells which have been exposed to a cancer antigen or a cancer-associated antigen in vitro. Lysates or 30 membrane fractions of dendritic cells may also be used as cancer vaccines. Dendritic cell vaccines are able to activate antigen-presenting cells directly. Other cancer vaccines

include ganglioside vaccines, heat-shock protein vaccines, viral and bacterial vaccines, and nucleic acid vaccines.

The use of immunostimulatory nucleic acids in conjunction with cancer vaccines provides an improved antigen-specific humoral and cell mediated immune response, in 5 addition to activating NK cells and endogenous dendritic cells, and increasing IFN $\alpha$  levels. This enhancement allows a vaccine with a reduced antigen dose to be used to achieve the same beneficial effect. In some instances, cancer vaccines may be used along with adjuvants, such as those described above.

As used herein, the terms "cancer antigen" and "tumor antigen" are used 10 interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in 15 normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by 20 viral genes such as those carried on RNA and DNA tumor viruses.

Other vaccines take the form of dendritic cells which have been exposed to cancer antigens *in vitro*, have processed the antigens and are able to express the cancer antigens at their cell surface in the context of MHC molecules for effective antigen presentation to other immune system cells.

25 The immunostimulatory nucleic acids are used in one aspect of the invention in conjunction with cancer vaccines which are dendritic cell based. A dendritic cell is a professional antigen presenting cell. Dendritic cells form the link between the innate and the acquired immune system by presenting antigens and through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local 30 environment. Dendritic cells efficiently internalize, process, and present soluble specific antigen to which it is exposed. The process of internalizing and presenting antigen causes rapid upregulation of the expression of major histocompatibility complex (MHC)

and costimulatory molecules, the production of cytokines, and migration toward lymphatic organs where they are believed to be involved in the activation of T cells.

Table D lists a variety of cancer vaccines which are either currently being used or are in development.

5

**Table D**

| Cancer Vaccines in Development or on the Market        |   |                              |
|--|---|------------------------------|
| MARKETER   | BRAND NAME (GENERIC NAME)   | INDICATION                   |
| Center of Molecular Immunology                         | EGF   | Cancer                       |
| Center of Molecular Immunology                         |   | Ganglioside cancer vaccine   |
| Center of Molecular Immunology                         | Anti-idiotypic  | Cancer vaccine               |
| ImClone Systems/Memorial Sloan-Kettering Cancer Center | Gp75 antigen  | Melanoma                     |
| ImClone Systems/Memorial Sloan-Kettering Cancer Center | Anti-idiotypic Abs  | Cancer vaccines              |
| Progenics Pharmaceuticals, Inc.                        | GMK melanoma vaccine  | Melanoma                     |
| Progenics Pharmaceuticals, Inc.                        | MGV ganglioside conjugate vaccine   | Lymphoma, colorectal, lung   |
| Corixa   | Her2/neu  | Breast, ovarian              |
| AltaRex  | Ovarex  | Ovarian                      |
| AVAX Technologies Inc.                                 | M-Vax, autologous whole cell  | Melanoma                     |
| AVAX Technologies Inc.                                 | O-Vax, autologous whole cell  | Ovarian                      |
| AVAX Technologies Inc.                                 | L-Vax, autologous whole cell  | Leukemia-AML                 |
| Biomira Inc./Chiron                                    | Theratope, STn-KLH  | Breast, Colorectal           |
| Biomira Inc.   | BLP25, MUC-1 peptide vaccine encapsulated in liposomal delivery system                  | Lung                         |
| Biomira Inc.   | BLP25, MUC-1 peptide vaccine encapsulated in liposomal delivery system + Liposomal IL-2 | Lung                         |
| Biomira Inc.   | Liposomal idiotypic vaccine   | Lymphoma B-cell malignancies |
| Ribi Immunochem  | Melaccine, cell lysate  | Melanoma                     |
| Corixa   | Peptide antigens, microsphere delivery system and LeIF adjuvant                         | Breast                       |
| Corixa   | Peptide antigens, microsphere delivery system and LeIF adjuvant                         | Prostate                     |
| Corixa   | Peptide antigens, microsphere delivery system and LeIF adjuvant                         | Ovarian                      |

|  |  |                 |
|--|--|-----------------|
| Corixa                                 | Peptide antigens, microsphere delivery system and LeIF adjuvant  | Lymphoma        |
| Corixa                                 | Peptide antigens, microsphere delivery system and LeIF adjuvant  | Lung            |
| Virus Research Institute               | Toxin/antigen recombinant delivery system  | All cancers     |
| Apollon Inc.                           | Genevax-TCR  | T-cell lymphoma |
| Bavarian Nordic Research Institute A/S | MVA-based (vaccinia virus) vaccine   | Melanoma        |
| BioChem Pharma/BioChem Vaccine         | PACIS, BCG vaccine   | Bladder         |
| Cantab Pharmaceuticals                 | TA-HPV   | Cervical        |
| Cantab Pharmaceuticals                 | TA-CIN   | Cervical        |
| Cantab Pharmaceuticals                 | DISC-Virus, immunotherapy  | Cancer          |
| Pasteur Mérieux Connaught              | Immucyst®/TheraCys® - BCG Immunotherapeutic (Bacillus Calmette-Guerin/Connaught), for intravesical treatment of superficial bladder cancer | Bladder         |

As used herein, chemotherapeutic agents embrace all other forms of cancer medicaments which do not fall into the categories of immunotherapeutic agents or cancer vaccines. Chemotherapeutic agents as used herein encompass both chemical and biological agents. These agents function to inhibit a cellular activity which the cancer cell is dependent upon for continued survival. Categories of chemotherapeutic agents include alkylating/alkaloid agents, antimetabolites, hormones or hormone analogs, and miscellaneous antineoplastic drugs. Most if not all of these agents are directly toxic to cancer cells and do not require immune stimulation. Combination chemotherapy and immunostimulatory nucleic acid administration increases the maximum tolerable dose of chemotherapy.

Chemotherapeutic agents which are currently in development or in use in a clinical setting are shown in Table E.

15

Table E  
*Cancer Drugs In Development or on the Market*

| Marketer | Brand Name       | Generic Name  | Indication                                       |
|----------|------------------|---------------|--|
| Abbott   | TNP 470/AGM 1470 | Fragyline     | Anti-Angiogenesis in Cancer                      |
| Takeda   | TNP 470/AGM 1470 | Fragyline     | Anti-Angiogenesis in Cancer                      |
| Scotia   | Meglamine GLA    | Meglamine GLA | Bladder Cancer                                   |
| Medeva   | Valstar          | Valrubicin    | Bladder Cancer - Refractory<br>in situ carcinoma |
| Medeva   | Valstar          | Valrubicin    | Bladder Cancer - Papillary                       |

|                     |                                    |                                    | Cancer  |
|---------------------|------------------------------------|------------------------------------|---|
| Rhone Poulenc       | Gliadel Wafer                      | Carmustaine + Polifepro Osan       | Brain Tumor   |
| Warner Lambert      | Undisclosed Cancer (b)             | Undisclosed Cancer (b)             | Cancer  |
| Bristol Myers Squib | RAS Farnesyl Transferase Inhibitor | RAS Farnesyl Transferase Inhibitor | Cancer  |
| Novartis            | MMI 270                            | MMI 270                            | Cancer  |
| Bayer               | BAY 12-9566                        | BAY 12-9566                        | Cancer  |
| Merck               | Farnesyl Transferase Inhibitor     | Farnesyl Transferase Inhibitor     | Cancer (Solid tumors - pancreas, colon, lung, breast) |
| Pfizer              | PFE                                | MMP                                | Cancer, angiogenesis                                  |
| Pfizer              | PFE                                | Tyrosine Kinase                    | Cancer, angiogenesis                                  |
| Lilly               | MTA/LY 231514                      | MTA/LY 231514                      | Cancer Solid Tumors                                   |
| Lilly               | LY 264618/Lometexol                | Lometexol                          | Cancer Solid Tumors                                   |
| Scotia              | Glamolec                           | LiGLA (lithium-gamma linolenate)   | Cancer, pancreatic, breast, colon                     |
| Warner Lambert      | CI-994                             | CI-994                             | Cancer, Solid Tumors / Leukemia                       |
| Schering AG         | Angiogenesis inhibitor             | Angiogenesis Inhibitor             | Cancer / Cardio                                       |
| Takeda              | TNP-470                            | n/k                                | Malignant Tumor                                       |
| Smithkline Beecham  | Hycamtin                           | Topotecan                          | Metastatic Ovarian Cancer                             |
| Novartis            | PKC 412                            | PKC 412                            | Multi-Drug Resistant Cancer                           |
| Novartis            | Valspodar                          | PSC 833                            | Myeloid Leukemia/Ovarian Cancer                       |
| Immunex             | Novantrone                         | Mitoxantrone                       | Pain related to hormone refractory prostate cancer.   |
| Warner Lambert      | Metaret                            | Suramin                            | Prostate  |
| Genentech           | Anti-VEGF                          | Anti-VEGF                          | Prostate / Breast / Colorectal / NSCL Cancer          |
| British Biotech     | Batimastat                         | Batimastat (BB94)                  | Pterygium   |
| Eisai               | E 7070                             | E 7070                             | Solid Tumors  |
| Biochem Pharma      | BCH-4556                           | BCH-4556                           | Solid Tumors  |
| Sankyo              | CS-682                             | CS-682                             | Solid Tumors  |
| Agouron             | AG2037                             | AG2037                             | Solid Tumors  |
| IDEC Pharma         | 9-AC                               | 9-AC                               | Solid Tumors  |
| Agouron             | VEGF/b-FGF Inhibitors              | VEGF/b-FGF Inhibitors              | Solid Tumors  |
| Agouron             | AG3340                             | AG3340                             | Solid Tumors / Macular Degen                          |
| Vertex              | Incel                              | VX-710                             | Solid Tumors - IV                                     |
| Vertex              | VX-853                             | VX-853                             | Solid Tumors - Oral                                   |
| Zeneca              | ZD 0101 (inj)                      | ZD 0101                            | Solid Tumors  |
| Novartis            | ISI 641                            | ISI 641                            | Solid Tumors  |
| Novartis            | ODN 698                            | ODN 698                            | Solid Tumors  |
| Tanabe Seiyaku      | TA 2516                            | Marimastat                         | Solid Tumors  |
| British Biotech     | Marimastat                         | Marimastat (BB 2516)               | Solid Tumors  |
| Celltech            | CDP 845                            | Aggrecanase Inhibitor              | Solid Tumors / Breast Cancer                          |
| Chiroscience        | D2163                              | D2163                              | Solid Tumors / Metastases                             |
| Warner Lambert      | PD 183805                          | PD 183805                          |   |
| Daiichi             | DX8951f                            | DX8951f                            | Anti-Cancer   |
| Daiichi             | Lemonal DP 2202                    | Lemonal DP 2202                    | Anti-Cancer   |
| Fujisawa            | FK 317                             | FK 317                             | Anticancer Antibiotic                                 |
| Chugai              | Picibanil                          | OK-432                             | Antimalignant Tumor                                   |
| Nycomed             | AD 32/valrubicin                   | Valrubicin                         | Bladder Cancer-Refractory                             |

| Amersham            |                      |                        | In situ Carcinoma                                      |
|---------------------|----------------------|------------------------|--|
| Nycomed Amersham    | Metastron            | Strontium Derivative   | Bone Cancer (adjunt therapy, Pain)                     |
| Schering Plough     | Temodal              | Temozolamide           | Brain Tumours  |
| Schering Plough     | Temodal              | Temozolamide           | Brain Tumours  |
| Liposome            | Evacet               | Doxorubicin, Liposomal | Breast Cancer  |
| Nycomed Amersham    | Yewtaxan             | Paclitaxel             | Breast Cancer Advanced, Ovarian Cancer Advanced        |
| Bristol Myers Squib | Taxol                | Paclitaxel             | Breast Cancer Advanced, Ovarian Cancer Advanced, NSCLC |
| Roche               | Xeloda               | Capecitabine           | Breast Cancer, Colorectal Cancer                       |
| Roche               | Furtulon             | Doxifluridine          | Breast Cancer, Colorectal Cancer, Gastric Cancer       |
| Pharmacia & Upjohn  | Adriamycin           | Doxorubicin            | Breast Cancer, Leukemia                                |
| Ivax                | Cyclopax             | Paclitaxel, Oral       | Breast/Ovarian Cancer                                  |
| Rhone Poulenc       | Oral Taxoid          | Oral Taxoid            | Broad Cancer   |
| AHP                 | Novantrone           | Mitoxantrone           | Cancer   |
| Sequus              | SPI-077              | Cisplatin, Stealth     | Cancer   |
| Hoechst             | HMR 1275             | Flavopiridol           | Cancer   |
| Pfizer              | CP-358, 774          | EGFR                   | Cancer   |
| Pfizer              | CP-609, 754          | RAS Oncogene Inhibitor | Cancer   |
| Bristol Myers Squib | BMS-182751           | Oral Platinum          | Cancer (Lung, Ovarian)                                 |
| Bristol Myers Squib | UFT (Tegafur/Uracil) | UFT (Tegafur/Uracil)   | Cancer Oral  |
| Johnson & Johnson   | Ergamisol            | Levamisole             | Cancer Therapy   |
| Giaxo Wellcome      | Eniluracil/776C85    | SFU Enhancer           | Cancer, Refractory Solid & Colorectal Cancer           |
| Johnson & Johnson   | Ergamisol            | Levamisole             | Colon Cancer   |
| Rhone Poulenc       | Campo                | Irinotecan             | Colorectal Cancer, Cervical Cancer                     |
| Pharmacia & Upjohn  | Camptosar            | Irinotecan             | Colorectal Cancer, Cervical Cancer                     |
| Zeneca              | Tomudex              | Raltrexed              | Colorectal Cancer, Lung Cancer, Breast Cancer          |
| Johnson & Johnson   | Leustain             | Cladribine             | Hairy Cell Leukaemia                                   |
| Ivax                | Paxene               | Paclitaxel             | Kaposi Sarcoma   |
| Sequus              | Doxil                | Doxorubicin, Liposomal | KS/Cancer  |
| Sequus              | Caelyx               | Doxorubicin, Liposomal | KS/Cancer  |
| Schering AG         | Fludara              | Fludarabine            | Leukaemia  |
| Pharmacia & Upjohn  | Pharmorubicin        | Epirubicin             | Lung/Breast Cancer                                     |
| Chiron              | DepoCyt              | DepoCyt                | Neoplastic Meningitis                                  |
| Zeneca              | ZD1839               | ZD 1839                | Non Small Cell Lung Cancer, Pancreatic Cancer          |
| BASF                | LU 79553             | Bis-Naphthalimide      | Oncology   |
| BASF                | LU 103793            | Dolastain              | Oncology   |
| Shering Plough      | Caetyx               | Doxorubicin-Liposome   | Ovarian/Breast Cancer                                  |
| Lilly               | Gemzar               | Gemcitabine            | Pancreatic Cancer, Non Small Cell Lung Cancer,         |

|                      |                                |                        |   |
|----------------------|--------------------------------|------------------------|---|
| Zeneca               | ZD 0473/Anormed                | ZD 0473/Anormed        | Breast, Bladder and Ovarian<br>Platinum based NSCL,<br>ovarian etc. |
| Yamanouchi           | YM 116                         | YM 116                 | Prostate Cancer   |
| Nycomed Amersham     | Seeds/I-125 Rapid St           | Lodine Seeds           | Prostate Cancer   |
| Agouron              | Cdk4/cdk2 inhibitors           | cdk4/cdk2 inhibitors   | Solid Tumors  |
| Agouron              | PARP inhibitors                | PARP Inhibitors        | Solid Tumors  |
| Chiroscience         | D4809                          | Dexifosamide           | Solid Tumors  |
| Bristol Myers Squib  | UFT (Tegafur/Uracil)           | UFT (Tegafur/Uracil)   | Solid Tumors  |
| Sankyo               | Krestin                        | Krestin                | Solid Tumors  |
| Asta Medica          | Ifex/Mesnex                    | Ifosamide              | Solid Tumors  |
| Bristol Meyers Squib | Ifex/Mesnex                    | Ifosamide              | Solid Tumors  |
| Bristol Meyers Squib | Vumon                          | Teniposide             | Solid Tumors  |
| Bristol Meyers Squib | Paraplatin                     | Carboplatin            | Solid Tumors  |
| Bristol Meyers Squib | Plantinol                      | Cisplatin, Stealth     | Solid Tumors  |
| Bristol Meyers Squib | Plantinol                      | Cisplatin              | Solid Tumors  |
| Bristol Meyers Squib | Vepeside                       | Etoposide              | Solid Tumors Melanoma   |
| Zeneca               | ZD 9331                        | ZD 9331                | Solid Tumors, Advanced Colorectal                                   |
| Chugai               | Taxotere                       | Docetaxel              | Solid Tumors, Breast Cancer   |
| Rhone Poulen         | Taxotere                       | Docetaxel              | Solid Tumors, Breast Cancer   |
| Glaxo Wellcome       | Prodrug of guanine arabinoside | Prodrug of arabinoside | T Cell Leukemia/Lymphoma & B Cell Neoplasm                          |
| Bristol Myers Squib  | Taxane Analog                  | Taxane Analog          | Taxol follow up   |

In one embodiment, the methods of the invention use immunostimulatory nucleic acids as a replacement to the use of IFN $\alpha$  therapy in the treatment of cancer. Currently, some treatment protocols call for the use of IFN $\alpha$ . Since IFN $\alpha$  is produced following the administration of some immunostimulatory nucleic acids, these nucleic acids can be used to generate IFN $\alpha$  endogenously.

The invention also includes a method for inducing antigen non-specific innate immune activation and broad spectrum resistance to infectious challenge using the immunostimulatory nucleic acids. The term antigen non-specific innate immune activation as used herein refers to the activation of immune cells other than B cells and for instance can include the activation of NK cells, T cells or other immune cells that can respond in an antigen independent fashion or some combination of these cells. A broad spectrum resistance to infectious challenge is induced because the immune cells are in

active form and are primed to respond to any invading compound or microorganism. The cells do not have to be specifically primed against a particular antigen. This is particularly useful in biowarfare, and the other circumstances described above such as travelers.

- 5        The stimulation index of a particular immunostimulatory nucleic acid can be tested in various immune cell assays. Preferably, the stimulation index of the immunostimulatory nucleic acid with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of  $^3\text{H}$  uridine in a murine B cell culture,
- 10      which has been contacted with 20  $\mu\text{M}$  of nucleic acid for 20h at 37°C and has been pulsed with 1  $\mu\text{Ci}$  of  $^3\text{H}$  uridine; and harvested and counted 4h later as described in detail in PCT Published Patent Applications PCT/US95/01570 (WO 96/02555) and PCT/US97/19791 (WO 98/18810) claiming priority to U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and October 30, 1997 respectively. For use *in*
- 15      *vivo*, for example, it is important that the immunostimulatory nucleic acids be capable of effectively inducing an immune response, such as, for example, antibody production.

Immunostimulatory nucleic acids are effective in non-rodent vertebrate.

- Different immunostimulatory nucleic acid can cause optimal immune stimulation depending on the type of subject and the sequence of the immunostimulatory nucleic acid. Many vertebrates have been found according to the invention to be responsive to the same class of immunostimulatory nucleic acids, sometimes referred to as human specific immunostimulatory nucleic acids. Rodents, however, respond to different nucleic acids. As shown herein an immunostimulatory nucleic acid causing optimal stimulation in humans may not generally cause optimal stimulation in a mouse and vice versa. An immunostimulatory nucleic acid causing optimal stimulation in humans often does, however, cause optimal stimulation in other animals such as cow, horses, sheep, etc. One of skill in the art can identify the optimal nucleic acid sequences useful for a particular species of interest using routine assays described herein and/or known in the art, using the guidance supplied herein.

- 30      The immunostimulatory nucleic acids may be directly administered to the subject or may be administered in conjunction with a nucleic acid delivery complex. A nucleic acid delivery complex shall mean a nucleic acid molecule associated with (e.g. ionically

- or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g., B cell surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, 5 virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.
- 10 Delivery vehicles or delivery devices for delivering antigen and nucleic acids to surfaces have been described. The Immunostimulatory nucleic acid and/or the antigen and/or other therapeutics may be administered alone (e.g., in saline or buffer) or using any delivery vehicles known in the art. For instance the following delivery vehicles have been described: Cochleates (Gould-Fogerite et al., 1994, 1996); Emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Mowat et al., 1993, Carlsson et al., 1991, Hu et., 1998, Morein et al., 1999); Liposomes (Childers et al., 1999, Michalek et al., 1989, 15 1992, de Haan 1995a, 1995b); Live bacterial vectors (e.g., *Salmonella*, *Escherichia coli*, *Bacillus calmette-guerin*, *Shigella*, *Lactobacillus*) (Hone et al., 1996, Pouwels et al., 1998, Chatfield et al., 1993, Stover et al., 1991, Nugent et al., 1998); Live viral vectors 20 (e.g., Vaccinia, adenovirus, Herpes Simplex) (Gallichan et al., 1993, 1995, Moss et al., 1996, Nugent et al., 1998, Flexner et al., 1988, Morrow et al., 1999); Microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); Nucleic acid vaccines (Fynan et al., 1993, Kuklin et al., 1997, Sasaki et al., 1998, Okada et al., 1997, Ishii et al., 1997); Polymers (e.g. 25 carboxymethylcellulose, chitosan) (Hamajima et al., 1998, Jabbal-Gill et al., 1998); Polymer rings (Wyatt et al., 1998); Proteosomes (Vancott et al., 1998, Lowell et al., 1988, 1996, 1997); Sodium Fluoride (Hashi et al., 1998); Transgenic plants (Tacket et al., 1998, Mason et al., 1998, Haq et al., 1995); Virosomes (Gluck et al., 1992, Mengiardi et al., 1995, Cryz et al., 1998); Virus-like particles (Jiang et al., 1999, Leibl et 30 al., 1998). Other delivery vehicles are known in the art and some additional examples are provided below in the discussion of vectors.

The term effective amount of a immunostimulatory nucleic acid refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a immunostimulatory nucleic acid for inducing mucosal immunity is that amount necessary to cause the development of IgA in response to an antigen upon 5 exposure to the antigen, whereas that amount required for inducing systemic immunity is that amount necessary to cause the development of IgG in response to an antigen upon exposure to the antigen. Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode 10 of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular immunostimulatory nucleic acid being administered, the antigen, the size of the subject, 15 or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular immunostimulatory nucleic acid and/or antigen and/or other therapeutic agent without necessitating undue experimentation.

Subject doses of the compounds described herein for mucosal or local delivery 20 typically range from about 0.1 µg to 10 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween. More typically mucosal or local doses range from about 10 µg to 5 mg per administration, and most typically from about 100 µg to 1 mg, with 2 - 4 administrations being spaced days or weeks apart. More typically, immune stimulant 25 doses range from 1 µg to 10 mg per administration, and most typically 10µg to 1 mg, with daily or weekly administrations. Subject doses of the compounds described herein for parenteral delivery for the purpose of inducing an antigen-specific immune response, wherein the compounds are delivered with an antigen but not another therapeutic agent are typically 5 to 10,000 times higher than the effective mucosal dose for vaccine 30 adjuvant or immune stimulant applications, and more typically 10 to 1,000 times higher, and most typically 20 to 100 times higher. Doses of the compounds described herein for parenteral delivery for the purpose of inducing an innate immune response or for

- increasing ADCC or for inducing an antigen specific immune response when the immunostimulatory nucleic acids are administered in combination with other therapeutic agents or in specialized delivery vehicles typically range from about 0.1 µg to 10 mg per administration, which depending on the application could be given daily, weekly, or
- 5 monthly and any other amount of time therebetween. More typically parenteral doses for these purposes range from about 10 µg to 5 mg per administration, and most typically from about 100 µg to 1 mg, with 2 - 4 administrations being spaced days or weeks apart. In some embodiments, however, parenteral doses for these purposes may be used in a range of 5 to 10,000 times higher than the typical doses described above.
- 10 For any compound described herein the therapeutically effective amount can be initially determined from animal models. A therapeutically effective dose can also be determined from human data for CpG oligonucleotides which have been tested in humans (human clinical trials have been initiated) and for compounds which are known to exhibit similar pharmacological activities, such as other mucosal adjuvants, e.g., LT
- 15 and other antigens for vaccination purposes, for the mucosal or local administration. Higher doses are required for parenteral administration. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the
- 20 ordinarily skilled artisan.
- The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.
- 25 For use in therapy, an effective amount of the immunostimulatory nucleic acid can be administered to a subject by any mode that delivers the nucleic acid to the desired surface, e.g., mucosal, systemic. Administering the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferred routes of administration include but are not limited to oral, parenteral,
- 30 intramuscular, intranasal, intratracheal, inhalation, ocular, vaginal, and rectal.

For oral administration, the compounds (i.e., immunostimulatory nucleic acids, antigens and other therapeutic agents) can be formulated readily by combining the active

- compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid 5 excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, 10 hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.
- 15 Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different 20 combinations of active compound doses.
- Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as 25 talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in 30 dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an

acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited  
5 to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for  
10 implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents,  
15 swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

The immunostimulatory nucleic acids and optionally other therapeutics and/or  
20 antigens may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to,  
25 those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid  
30 and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03%

w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of a Immunostimulatory nucleic acid and optionally antigens and/or other therapeutic agents optionally included in a pharmaceutically-acceptable carrier. The term pharmaceutically-acceptable carrier means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term carrier denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

The immunostimulatory nucleic acids useful in the invention may be delivered in mixtures with additional adjuvant(s), other therapeutics, or antigen(s). A mixture may consist of several adjuvants in addition to the Immunostimulatory nucleic acid or several antigens or other therapeutics.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular adjuvants or antigen selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration,

- purpose of the immunization (i.e., prophylactic or therapeutic), nature and severity of the disorder, age and body weight of the patient, different doses may be necessary. The administration of a given dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units. Multiple 5 administration of doses at specific intervals of weeks or months apart is usual for boosting the antigen-specific responses.
- Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of 10 release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include 15 non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di-and tri-glycerides; hydrogel release systems; sylvatic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent 20 of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware 25 delivery systems can be used, some of which are adapted for implantation.
- The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

30

Examples

*Materials and Methods:*

Oligodeoxynucleotides: Native phosphodiester and phosphorothioate-modified ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). ODN were tested for endotoxin using the LAL-assay (LAL-assay BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml). For *in vitro* assays, ODN were diluted in TE-buffer (10 mM Tris, pH 7.0, 1 mM EDTA), and stored at -20°C. For *in vivo* use, ODN were diluted in phosphate buffered saline (0.1 M PBS, pH 7.3) and stored at 4°C. All dilutions were carried out using pyrogen-free reagents.

Isolation of human PBMC and cell culture: Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) as described (Hartmann et al., 1999 Proc. Natl. Acad. Sci USA 96:9305-10). Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, NY) (complete medium). Cells (final concentration  $1 \times 10^6$  cells/ml) were cultured in complete medium in a 5% CO<sub>2</sub> humidified incubator at 37°C. ODN and LPS (from *Salmonella typhimurium*, Sigma Chemical Co., St. Louis, MO) or anti-IgM were used as stimuli. For measurement of human NK lytic activity, PBMC were incubated at  $5 \times 10^6$ /well in 24-well plates. Cultures were harvested after 24 hours, and cells were used as effectors in a standard 4 hours <sup>51</sup>Cr-release assay against K562 target cells as previously described (Ballas et al., 1996 J. Immunol. 157:1840-1845). For B cell proliferation, 1 µCi of <sup>3</sup>H thymidine was added 18 hours before harvest, and the amount of <sup>3</sup>H thymidine incorporation was determined by scintillation counting at day 5. Standard deviations of the triplicate wells were < 5%.

Flow cytometry on human PBMC: Surface antigens on primate PBMC were stained as previously described (Hartmann et al., 1998 J. Pharmacol. Exp. Ther. 285:920-928). Monoclonal antibodies to CD3 (UCHT1), CD14 (M5E2), CD19 (B43), CD56 (B159), CD69 (FN50) and CD86 (2331 [FUN-1]) were purchased from Pharmingen, San Diego, CA. IgG<sub>1</sub>,κ (MOPC-21) and IgG<sub>2b</sub>,κ (Hartmann et al., 1999 Proc. Natl. Acad. Sci USA 96:9305-10) were used to control for non-specific staining. NK cells were identified by CD56 expression on CD3, CD14 and CD19 negative cells, whereas B cells

were identified by expression of CD19. Flow cytometric data of 10000 cells per sample were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA). The viability of cells within the FSC/SSC gate used for analysis was examined by propidium iodide staining (2 µg/ml) and found to be higher than 98%. Data were  
5 analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stanford, CA).

*Results:*

*Example 1: CpG-dependent stimulation of human B cells depends on methylation and ODN length.*

10 Human PBMC were obtained from normal donors and cultured for five days at 2 × 10<sup>5</sup> cells/well with the indicated concentrations of the indicated ODN sequences. As shown in Table F, human PBMCs proliferate above the background when cultured with a variety of different CpG ODN, but also show some proliferation even with ODN that do not contain any CpG motifs. The importance of unmethylated CpG motifs in providing  
15 optimal immune stimulation with these ODN is demonstrated by the fact that ODN 1840 (SEQ ID NO. 83) induces 56,603 counts of <sup>3</sup>H-thymidine incorporation whereas the same T-rich ODN with the CpG motifs methylated (non-CpG), 1979 (SEQ ID NO. 222), induces lower, but still increased over background, activity (only 18,618 counts) at the same concentration of 0.6 µg/ml. The reduced proliferation at higher ODN  
20 concentrations may be an artifact of the cells becoming exhausted under these experimental conditions or could reflect some toxicity of the higher ODN concentrations. Interestingly, shorter ODN containing CpG motifs, such as the 13-14 mers 2015 and 2016, are less stimulatory despite the fact that their molar concentration would actually  
25 be higher since the ODNs were added on the basis of mass rather than molarity. This demonstrates that ODN length may also be an important determinant in the immune effects of the ODN. A non-CpG ODN but slight T-rich ODN (about 30% T), 1982 (SEQ ID NO. 225), caused only a small amount of background cell proliferation.

Table F

| Oligo Concentration |            |           |         |
|---------------------|------------|-----------|---------|
| ODN#                | 0.15 µg/ml | 0.6 µg/ml | 2 µg/ml |

| Cues only                | 648  | 837    | 799    |
|--------------------------|------|--------|--------|
| 1840<br>(SEQ ID NO. 83)  | 5744 | 56,603 | 31,787 |
| 2016<br>(SEQ ID NO. 256) | 768  | 4607   | 20,497 |
| 1979<br>(SEQ ID NO. 222) | 971  | 18,618 | 29,246 |
| 1892<br>(SEQ ID NO. 135) | 787  | 10,078 | 22,850 |
| 2010<br>(SEQ ID NO. 250) | 849  | 20,741 | 8,054  |
| 2012<br>(SEQ ID NO. 252) | 2586 | 62,955 | 52,462 |
| 2013<br>(SEQ ID NO. 253) | 1043 | 47,960 | 47,231 |
| 2014<br>(SEQ ID NO. 254) | 2700 | 50,708 | 46,625 |
| 2015<br>(SEQ ID NO. 255) | 1059 | 23,239 | 36,119 |

Numbers represent cpm of  $^3\text{H}$ -thymidine incorporation for cultures of human PBMCs set up as described above.

*Example 2. Concentration-dependent activation of human NK cell activity with thymidine-rich ODN.*

- 5 Human PBMCs were cultured for 24 hours with a panel of different CpG or non-CpG ODN at two different concentrations, and then tested for their ability to kill NK target cells as described previously (Ballas et al., 1996 J. Immunol. 157:1840-1845). Killing is measured as lytic units, or L.U. The human donor used in this experiment had a background level of 3.69 L.U. which increased to 180.36 L.U. using the positive control, IL-2. A CpG oligo, 2006 (SEQ ID NO. 246), induced high levels of NK lytic function at a low concentration of 0.6, and a lower level at a concentration of 6.0. Surprisingly, a T-rich ODN in which the CpG motifs of 2006 were methylated (ODN at

2117 (SEQ ID NO. 358)) or inverted to GpCs (ODN 2137 (SEQ ID NO. 886) retained strong immune stimulatory function at the higher ODN concentrations, as shown in Table G. These concentration-dependent immune stimulatory effects are not a general property of the phosphorothioate backbone since the experiments described below demonstrate that a poly-A ODN, is nonstimulatory above background levels. Some stimulation is seen with a 24-base long ODN in which all of the base positions are randomized so that A, C, G, and T will occur at a frequency of 25% in each of the base positions (ODN 2182 (SEQ ID NO. 432)). However, the stimulatory effect of such a 24-base ODN is greatly enhanced if it is pure poly-T, in which case stimulation is also seen at the lowest concentration of 0.6 µg/ml (ODN 2183 (SEQ ID NO. 433)). In fact, the stimulatory activity of ODN SEQ ID NO. 433 at this low concentration is higher than that of any other ODN tested at this low concentration, aside from the optimal human immune stimulatory ODN of SEQ ID NO. 246. In fact, the higher concentration of ODN SEQ ID NO. 433 stimulated more NK activity than any other phosphorothioate ODN except for the strong CpG ODN 2142 (SEQ ID NO. 890), which was marginally higher. If the G content of ODN SEQ ID NO. 246 is increased relative to the T content by addition of more Gs, thus resulting in a decrease in the proportion of T nucleotides the immune stimulatory effect of the ODN is reduced (see ODN 2132 (SEQ ID NO. 373)). Thus, the T content of an ODN is an important determinant of its immune stimulatory effect. Although a poly-T ODN is the most stimulatory of the non-CpG ODN, other bases are also important in determining the immune stimulatory effect of a non-CpG ODN. ODN 2131 (SEQ ID NO. 372), in which slightly more than half of the bases are T and in which there are no Gs, is immune stimulatory at a concentration of 6 µg/ml but has less activity than other T-rich ODN. If the 6 A's in ODN 2131 (SEQ ID NO. 372) are replaced by 6 Gs, the immune stimulatory effect of the ODN can be increased (see ODN 2130 (SEQ ID NO. 371)).

Table G

*HUMAN PBL CULTURED OVERNIGHT WITH OLIGOS*

| SR<br>%SR                | 256<br>7.11 | 0.63  | 1.25  | 2.50   | 5.00  | 10.00 | 20.00  | L.U. |
|--------------------------|-------------|-------|-------|--------|-------|-------|--------|------|
| EFFECTOR<br>CONTROL [RM] |             |       |       |        |       |       |        |      |
| ALONE                    |             | 2.65  | 5.45  | 10.15  | 17.65 | 29.92 | 39.98  | 3.69 |
| + IL2 (100 U/ml)         | 35.95       | 57.66 | 86.26 | 100.39 | 99.71 | 93.64 | 180.36 |      |
| + 1585 (0.6 ug/ml)       | 3.75        | 6.10  | 12.14 | 23.70  | 36.06 | 43.98 | 5.48   |      |
| + 1585 (6.0 ug/ml)       | 15.42       | 31.09 | 47.07 | 73.34  | 94.29 | 97.73 | 35.85  |      |
| + 2006 (0.6 ug/ml)       | 6.71        | 15.99 | 26.92 | 44.75  | 64.12 | 68.83 | 16.96  |      |
| + 2006 (6.0 ug/ml)       | 6.19        | 8.18  | 16.13 | 24.35  | 39.35 | 56.07 | 8.04   |      |
| + 2117 (0.6 ug/ml)       | 4.54        | 4.73  | 9.56  | 18.04  | 28.57 | 39.85 | 3.49   |      |
| + 2117 (6.0 ug/ml)       | 7.03        | 10.76 | 16.90 | 30.59  | 52.14 | 59.46 | 10.96  |      |
| + 2137 (0.6 ug/ml)       | 4.61        | 5.35  | 10.04 | 15.16  | 23.79 | 37.86 | 2.57   |      |
| + 2137 (6.0 ug/ml)       | 7.99        | 10.37 | 16.55 | 32.32  | 49.78 | 60.30 | 11.01  |      |
| + 2178 (0.6 ug/ml)       | 2.88        | 4.52  | 11.47 | 16.05  | 24.85 | 34.27 | 2.37   |      |
| + 2178 (6.0 ug/ml)       | 4.21        | 5.03  | 11.16 | 16.39  | 28.22 | 36.45 | 2.94   |      |
| + 2182 (0.6 ug/ml)       | 2.42        | 6.57  | 10.49 | 19.73  | 26.55 | 35.30 | 2.89   |      |
| + 2182 (6.0 ug/ml)       | 4.11        | 7.98  | 14.60 | 26.56  | 40.40 | 51.98 | 7.59   |      |
| + 2183 (0.6 ug/ml)       | 3.73        | 8.46  | 15.52 | 24.48  | 37.78 | 56.77 | 7.80   |      |
| + 2183 (0.6 ug/ml)       | 8.86        | 12.89 | 23.08 | 41.49  | 66.26 | 75.85 | 16.57  |      |
| + 2140 (0.6 ug/ml)       | 3.78        | 5.27  | 12.30 | 20.79  | 35.75 | 45.62 | 5.40   |      |
| + 2140 (6.0 ug/ml)       | 6.56        | 13.24 | 21.26 | 37.96  | 60.80 | 73.05 | 14.82  |      |

|                       |      |       |       |       |       |       |       |
|-----------------------|------|-------|-------|-------|-------|-------|-------|
| + 2141 (0.6<br>ug/ml) | 2.63 | 6.34  | 10.21 | 17.73 | 30.93 | 43.57 | 4.29  |
| + 2141 (6.0<br>ug/ml) | 4.98 | 15.30 | 25.22 | 37.88 | 58.47 | 69.12 | 14.83 |
| + 2142 (0.6<br>ug/ml) | 3.18 | 3.66  | 6.99  | 14.62 | 19.68 | 32.52 | 1.56  |
| + 2142 (6.0<br>ug/ml) | 7.08 | 15.80 | 25.65 | 41.72 | 68.09 | 73.14 | 17.11 |
| + 2143 (0.6<br>ug/ml) | 4.12 | 6.90  | 10.77 | 22.96 | 35.78 | 42.94 | 5.19  |
| + 2143 (6.0<br>ug/ml) | 3.16 | 8.40  | 12.38 | 21.69 | 34.80 | 54.21 | 6.64  |
| + 2159 (6.0<br>ug/ml) | 5.05 | 11.76 | 21.67 | 41.12 | 51.68 | 65.47 | 13.19 |
| + 2132 (6.0<br>ug/ml) | 4.23 | 6.06  | 10.50 | 18.74 | 32.68 | 44.06 | 4.61  |
| + 2179 (6.0<br>ug/ml) | 6.14 | 9.49  | 21.06 | 42.48 | 60.12 | 71.87 | 14.54 |
| + 2180 (6.0<br>ug/ml) | 2.37 | 8.57  | 15.44 | 29.66 | 44.35 | 61.31 | 9.47  |
| + 2133 (6.0<br>ug/ml) | 6.53 | 12.58 | 23.10 | 38.03 | 61.16 | 68.36 | 14.62 |
| + 2134 (6.0<br>ug/ml) | 7.51 | 12.14 | 21.14 | 32.46 | 54.47 | 67.12 | 12.98 |
| + 2184 (6.0<br>ug/ml) | 5.22 | 9.19  | 17.54 | 30.76 | 45.35 | 63.55 | 10.42 |
| + 2185 (6.0<br>ug/ml) | 8.11 | 14.77 | 26.27 | 40.31 | 55.61 | 70.65 | 15.60 |
| + 2116 (6.0<br>ug/ml) | 5.58 | 10.54 | 16.77 | 37.82 | 59.80 | 66.33 | 13.07 |
| + 2181 (6.0<br>ug/ml) | 4.43 | 9.85  | 17.55 | 27.05 | 53.16 | 69.16 | 11.43 |
| + 2130 (6.0<br>ug/ml) | 3.81 | 8.07  | 17.11 | 27.17 | 42.04 | 53.73 | 8.27  |
| + 2131 (6.0<br>ug/ml) | 2.29 | 6.73  | 7.30  | 18.02 | 32.73 | 49.06 | 5.08  |
| + 2156 (0.3<br>ug/ml) | 2.50 | 5.26  | 8.20  | 15.95 | 26.64 | 33.07 | 2.31  |
| + 2156 (1.0<br>ug/ml) | 5.91 | 10.99 | 17.31 | 26.97 | 50.64 | 63.78 | 10.84 |
| + 2157 (0.3<br>ug/ml) | 2.36 | 4.00  | 6.65  | 12.94 | 24.13 | 38.86 | 2.58  |

|                    |      |      |       |       |       |       |       |
|--------------------|------|------|-------|-------|-------|-------|-------|
| + 2157 (1.0 ug/ml) | 3.72 | 9.55 | 17.15 | 34.55 | 52.27 | 65.33 | 11.58 |
| + 2158 (0.3 ug/ml) | 1.25 | 2.36 | 6.90  | 16.39 | 15.63 | 29.82 | 1.17  |
| + 2158 (1.0 ug/ml) | 4.73 | 7.26 | 11.07 | 15.55 | 30.80 | 43.71 | 4.16  |
| + 2118 (0.6 ug/ml) | 1.55 | 3.38 | 6.85  | 13.36 | 20.15 | 27.71 | 1.13  |
| + 2118 (6.0 ug/ml) | 2.65 | 3.88 | 9.29  | 12.19 | 22.47 | 28.99 | 1.34  |

*Example 3: Induction of B cell proliferation by T-rich non-CpG ODN.*

To assess the ability of T-rich ODN to activate B cell proliferation, human PBMCs were stained with the cytoplasmic dye CSFE, incubated with five days with the indicated ODN at either 0.15 or 0.3 ug/ml, and then analyzed by flow cytometry. B cells were identified by gating on cells positive for the lineage marker CD19. CpG ODN 2006 was a strong inducer of B cell proliferation, and this effect was reduced if the CpG motifs were methylated or inverted to GpC as shown in Figure 1 at an ODN concentration of 0.3 ug/ml. The base composition of the ODN appears to be important in determining the immune stimulatory effect. Reducing the T content of an ODN substantially reduces immune stimulatory effect, as exemplified by ODN 2177 (SEQ ID NO. 427) in which 6 of the Ts present in ODN 2137 (SEQ ID NO. 886) have been switched to A's, resulting in a greatly reduced immune stimulatory effect. The importance of T's in the immune stimulatory effect of an ODN is also shown by comparison of ODN 2116 (SEQ ID NO. 357) and 2181 (SEQ ID NO. 431), which differ in the 3' end of the ODN. ODN 2181, in which the 3' end is poly-T is more stimulatory than ODN 2116, in which the 3' end is poly-C, despite the fact that both ODN have a TCGTCG at the 5' end.

*Example 4: B Cell Proliferation Induced by TG Oligonucleotides*

The stimulatory effects of TG motifs are shown in Figure 2. ODN 2137 has the identical base composition as ODN 2006, but the CG motifs have all been inverted to GC's resulting in a CG-free nucleic acid. ODN does however contain 6 TG

dinucleotides. In ODN 2177, all the TG dinucleotides of ODN 2137 have been changed to AG. Although ODN 2177 contains only 6 adenines, it is virtually nonstimulatory at a concentration of 0.2 µg/ml. For comparison, an ODN 24 bases in length in which each position is randomized to be any of the four bases (ODN 2182) induces > 12% of B cells 5 to proliferate at a concentration of 0.2 µg/ml. These results indicate that the stimulatory effects of ODN 2137 are not simply those of a phosphorothioate backbone, but relate to the presence of TG dinucleotides.

In order to determine the effect of varying the number of TG dinucleotide motifs, ODN 2200 and ODN 2202 were compared, as shown in Figure 2. Both ODN contain 18 10 Ts and 6 Gs, but in ODN 2200 all of the Gs are consecutive, so that there is only one TG dinucleotide, whereas in ODN 2202, the Gs are split up as GG dinucleotides throughout the ODN so that there are three TGs. ODN 2202 is significantly more stimulatory than ODN 2200, consistent with the model that at least three TG motifs in an ODN are required for optimal stimulatory activity. It is likely that even higher levels of 15 stimulation could be achieved if the TG motifs had been optimized as taught herein.

*Example 5: Effects of TTG versus TTG motifs.*

Figure 3 shows the results of experiments conducted to study TG content in terms of the relative levels of Ts versus Gs as it relates to the stimulatory effect of an ODN. The Figure shows that an ODN in which all of the bases are randomized to be either T or 20 G (ODN 2188 (SEQ ID NO. 905)) is nonstimulatory at a concentration of 0.2 µg/ml, similar to an ODN in which all of the bases are randomized to be either A or G (ODN 2189 (SEQ ID NO. 906)). However, at the higher concentration of 2 µg/ml, the randomized T/G ODN 2188 is significantly more stimulatory. This latter level of stimulation is still lower than that which occurs with a totally randomized ODN (ODN 25 2182 (SEQ ID NO. 432)). The highest stimulation at low concentrations is seen with an ODN in which half of the bases are fixed at T and the other half of the bases are randomized to be either T or G (ODN 2190 (SEQ ID NO. 907)). Since every other base is fixed to be a T, there cannot be any TG motifs. The data in Figure 3 show that increasing the TG content of an ODN improves its stimulatory activity.

In yet other experiments, the results of which are not diagrammed herein, ODN 2190 (SEQ ID NO. 907) exhibited a stimulation of NK activity compared to ODN 2188 (SEQ ID NO. 905) or ODN 2189 (SEQ ID NO. 906).

**Examples 6-8**

5

*Introduction:*

Above, we demonstrated that Poly T sequences are able to enhance stimulation of B and NK cells. Here and below we investigate the effect of a variety of non-CpG T-rich ODN as well as Poly C ODN for their ability to stimulate human B cells, NK cells and monocytes.

10

*Materials and Methods:*

15

**Oligonucleotides:** Phosphorothioate-modified ODN were purchased from ARK Scientific GmbH (Darmstadt, Germany). The sequences used were: 1982: 5'-tccaggactcttcaggtt-3' (SEQ ID NO.: 225), 2006: 5'-tcgtcgtttgcgttttgtcgtt-3' (SEQ ID NO.: 246), 2041: 5'-ctggctttctgggtttttctgg-3' (SEQ ID NO.: 282), 2117: 5'-tzgtzgttttgztgztgtzgtt-3' (SEQ ID NO.: 358), 2137: 5'-tgctgctttgtgcctttgtgcct-3' (SEQ ID NO.: 886), 2183: 5'-tttttttttttttttttt-3' (SEQ ID NO.: 433), 2194: 5'-tttttttttttttttttttt-3' (SEQ ID NO.: 911), 2196: 5'-tttttttttttttt-3' (SEQ ID NO.: 913), 5126: 5'-ggttctttggtccttgtct-3' (SEQ ID NO.: 1058), 5162: 5'-tttttttttttttttttttt-3' (SEQ ID NO.: 1094), 5163: 5'-aaaaaaaaaaaaaaaaaaaaaaaaaaa-3' (SEQ ID NO.: 1095), 5168: 5'-ccccccccccccccccccccccccccc-3' (SEQ ID NO.: 1096) and 5169: 5'-cgcgcgcgcgcgcgcgcgcgcg-3' (SEQ ID NO.: 1097). Most ODN were tested for LPS content using the LAL assay (Bio Whittaker, Belgium) (lower detection limit 0.1EU/ml) also described herein. For all assays ODN were diluted in TE buffer and stored at -20°C. All dilutions were conducted using pyrogen-free reagents.

20

**Cell preparation and cell culture:** Human PBMC were isolated from peripheral blood of healthy volunteers, obtained by the German Red Cross (Ratingen, Germany), as described above in Example 1, but all material were purchased from Life Technologies, Germany and were endotoxin-tested. For the B cell, NK cell and monocyte activation assays PBMC were cultured in complete medium at a concentration of  $2 \times 10^6$  cells/ml in

200 $\mu$ l in 96 round bottom plates in a humidified incubator at 37°C. Different ODNs, LPS (Sigma) or IL-2 (R&D Systems, USA) were used as stimuli. At the indicated time points, cells were harvested for flow cytometry.

Flow cytometry: MAbs used for staining of surface antigens were: CD3, CD14,  
5 CD19, CD56, CD69, CD80 and CD86 (all obtained from Pharmingen/Becton Dickinson, Germany). For monocytes Fc receptors were blocked using human IgG (Mylenyi, Germany) as previously described (Bauer, M., K. Heeg, H. Wagner, and G. B. Lipford. 1999. DNA activates human immune cells through a CpG sequence dependent manner. *Immunology* 97:699). Flow cytometric data of at least 1000 cells of a specified  
10 subpopulation (B cells, monocytes, NK cells, NKT cells or T cells) were acquired on a FACSCalibur (Becton Dickinson). Data were analyzed using the program CellQuest (Becton Dickinson).

NK-mediated cytotoxicity: PBMC were cultured overnight with or without 6 $\mu$ g/ml ODN or 100U/ml IL-2 at 37°C, 5% CO<sub>2</sub>. The next morning, K-562 target cells  
15 were labeled with a fluorescent dye, CFSE, as described previously for human B cells (Hartmann, G., and A. M. Krieg. 2000. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* 164:944). PBMC were added in different ratios (50:1, 25:1 and 12.5:1) to 2x10<sup>5</sup> target cells and incubated for 4h at 37°C. Cells were harvested and incubated with the DNA-specific dye 7-AAD (Pharmingen) for  
20 detection of apoptotic cells. Results were measured by flow cytometry.

ELISA: PBMC (3x10<sup>6</sup> cells/ml) were cultured with the specified concentrations of ODN or LPS for 24h (IL-6, IFN $\gamma$  and TNF $\alpha$ ) or 8h (IL-1 $\beta$ ) in 48 well plates in a humidified atmosphere at 37°C. Supernatants were collected and cytokines were measured using OPTeia ELISA Kits (Pharmingen) for IL-6, IFN $\gamma$  and TNF $\alpha$  or an Eli-  
25 pair ELISA assay (Hoelzel, Germany) for IL-1 $\beta$  according to the manufacturer protocols.

*Example 6: B cell activation induced by ODNs lacking CpG motifs*

In the Experiments described above in Example 3, we demonstrate that T-rich ODN were capable of activating B cells. We expand those studies here using additional ODN and different cell and reagent sources. In a first set of experiments, we compared  
30 the activation potential of different non-CpG T-rich ODNs with the very potent known CpG ODN 2006 (SEQ ID NO.: 246). PBMC (2x10<sup>6</sup> cells/ml) of a blood donor (n=2) were incubated with the indicated concentrations of ODNs 2006 (SEQ ID NO.: 246),

2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 5126 (SEQ ID NO.: 1058), and 5162 (SEQ ID NO.: 1094). Cells were incubated for 48h at 37°C as described above and stained with mAb for CD19 (B cell marker) and CD86 (B cell activation marker, B7-2). Expression was measured by flow cytometry.

5       Using different concentrations of ODNs, we showed (Fig. 4) that T-rich ODNs without a CpG motif, can induce stimulation of human B cells. ODN 5126 (SEQ ID NO.: 1058) which contains only a single poly-T sequence but is greater than 50% T, caused high levels of human B cell activation. Although there are some similarities to SEQ ID NO.: 246 (e.g. more than 80% T/G content), this ODN clearly lacks any known  
10 immunostimulatory CpG motif. Surprisingly, for all tested T-rich ODNs, the highest stimulatory index was obtained at concentrations between 3 and 10 $\mu$ g/ml. The highest stimulatory index of the tested ODNs was achieved by CpG/T-rich ODN SEQ ID NO.: 246 at 0.4 $\mu$ g/ml. Interestingly, the activity decreased at high concentrations.

Poly A, Poly C and Poly T sequences were synthesized and tested for biological 15 activity. PBMC ( $2 \times 10^6$  cells/ml) of one representative donor (n=3) were stimulated as described above by 0.4 $\mu$ g/ml, 1.0 $\mu$ g/ml or 10.0 $\mu$ g/ml of the following ODNs: 2006 (SEQ ID NO.: 246), 2196 (SEQ ID NO.: 913) (Poly T, 18 bases), 2194 (SEQ ID NO.: 911) (Poly T, 27 bases), 5162 (SEQ ID NO.: 1094) (Poly T, 30 bases), 5163 (SEQ ID NO.: 1095) (Poly A, 30 bases), 5168 (SEQ ID NO.: 1096) (Poly C, 30 bases) and 5169 (SEQ 20 ID NO.: 1097) (Poly CG, 30 bases). Expression of the activation marker CD86 (B7-2) on CD19-positive B cells was measured by flow cytometry.

Fig. 5 demonstrates that the length of the sequence, at least for Poly T ODNs, has an important impact on its activity. A Poly T sequence containing only 18 bases (SEQ ID NO.: 913) was shown to be less stimulatory than one with 27 bases (SEQ ID NO.: 911) or one with 30 bases (SEQ ID NO.: 1094) with a clear rank of stimulation: SEQ ID NO.: 1094 > SEQ ID NO.: 911 > SEQ ID NO.: 913. Poly A (SEQ ID NO.: 1095) or Poly CG (SEQ ID NO.: 1097) sequences, in contrast, do not induce activation of human B cells. Surprisingly it was also discovered that Poly C sequences (SEQ ID NO.: 1096) can activate human B cells at least at high concentrations (10 $\mu$ g/ml) (Fig. 5).

30       Two other T-rich ODNs, namely 1982 (SEQ ID NO.: 225) and 2041 (SEQ ID NO.: 282) lacking CpG motifs were tested for their effect on human B cells. PBMC (n=2) were incubated with the indicated concentrations of ODN 2006 (SEQ ID NO.:

246), 1982 (SEQ ID NO.: 225) and 2041 (SEQ ID NO.: 282) as described above. B cell activation (expression of the activation marker CD86) was measured by flow cytometry.

Fig. 6 demonstrates that T-rich non-CpG ODN are immunostimulatory at concentrations higher than 1 µg/ml. Incorporation of a CpG motif into 1982 enhanced the 5 immunostimulatory activity. Elongation with a Poly T sequence did not enhance the immunostimulatory activity of this already T-rich ODN but rather, decreased the activation potential slightly.

*Example 7: Immunostimulation of non-CpG ODNs is reflected in the enhancement of NK activation, NK cytotoxicity and monocyte activation*

10 NK cells as well as monocytes were tested for their response to non-CpG ODNs. PBMC ( $2 \times 10^6$  cells/ml) were incubated with 6 µg/ml of the following ODNs (n=4): 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058). After 24h of cultivation at 37°C cells were harvested and stained with mAb for CD3 (T cell marker), 15 CD56 (NK cell marker) and CD69 (early activation marker) as described above. Expression of CD69 on CD56-positive NK cells was measured by flow cytometry.

Fig. 7 shows that for Poly T ODNs similar effects can be observed as described in Fig. 5. The stimulation of NK cells, like B cells, may be influenced by the length of the ODN. ODN 2183 (SEQ ID NO.: 433) (21 bases) induced activation of NK cells but to a lesser extent than the longer ODN 2194 (SEQ ID NO.: 911) (27 bases), as measured by enhanced expression of the early activation marker CD69. ODN 5126 (SEQ ID NO.: 1058) was also demonstrated to activate human NK cells (Fig. 7).

It is believed that the anti-tumor activity of CpG ODNs can be assessed by the ability of the ODN to enhance NK-mediated cytotoxicity *in vitro*. ODNs containing at 25 the 5' and 3' ends stretches of Poly G were shown to result in the highest induction of cytotoxicity (Ballas, Z. K., W. L. Rasmussen, and A. M. Krieg. 1996. Induction of natural killer cell activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 157:1840). To investigate the influence of non-CpG T-rich ODN on NK cytotoxicity, we analyzed the effect of the 30 ODNs 2194 (SEQ ID NO.: 911) and 5126 (SEQ ID NO.: 1058) on NK-mediated lysis

(Fig. 8). NK-mediated lysis of K-562 target cells was measured after over night incubation of PBMC with 6 $\mu$ g/ml of the ODN 2006 (SEQ ID NO.: 246), SEQ ID NO.: 911 (SEQ ID NO.: 911) (Poly T, 27 bases) and 5126 (SEQ ID NO.: 1058) as described above. SEQ ID NO.: 1058 demonstrated small increases in lysis by human NK cells as compared to no ODN. SEQ ID NO.: 911 and SEQ ID NO.: 246 enhanced human NK cell cytotoxicity to an even higher extent.

Previous reports demonstrated that not only NK cells but also NKT cells are mediators of cytotoxic responses to tumor cells (14). We, therefore, looked at the potential activation of human NKT cells by T-rich non-CpG ODN. PBMC of one representative donor (n=2) were incubated with 6 $\mu$ g/ml ODN 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 913) and 5126 (SEQ ID NO.: 1058) for 24h as described above. Activation of NKT cells was measured by flow cytometry after staining of cells with mAb for CD3 (T cell marker), CD56 (NK cell marker) and CD69 (early activation marker). Shown is the expression of CD69 on CD3 and CD56 double-positive cells (NKT cells).

In Fig. 9, SEQ ID NO.: 911 as well as SEQ ID NO.: 1058 were found to stimulate NKT cells. Similar to NK cells SEQ ID NO.: 911 (Poly T) was more active than SEQ ID NO. 1058. In addition, as described above for B cells and NK cells, the length of the ODN has some influence on the immunostimulatory potential, with the longer ODN having stronger effects on NKT cells. Similar results were observed for human T cells.

Another type of cell of the immune system involved in fighting infections is the monocytes. These cells release upon activation a variety of cytokines and can mature into dendritic cells (DC), professional antigen-presenting cells (Roitt, I., J. Brostoff, and D. Male. 1998. *Immunology*. Mosby, London). Fig. 10 shows activation of human monocytes after culturing of PBMC with different ODNs. PBMC ( $2 \times 10^6$  cells/ml) were incubated with 6 $\mu$ g/ml 2006 (SEQ ID NO.: 246), 2117 (SEQ ID NO.: 358), 2137 (SEQ ID NO.: 886), 2178 (SEQ ID NO.: 1096), 2183 (SEQ ID NO.: 433), 2194 (SEQ ID NO.: 911), 5126 (SEQ ID NO.: 1058) and 5163 (SEQ ID NO.: 1095) overnight at 37°C as described above (n=3). Cells were harvested and stained for CD14 (monocyte marker) and CD80 (B7-1, activation marker). Expression was measured by flow cytometry.

As demonstrated above for NK and B cells, T-rich sequences (e.g., SEQ ID NO.: 433, SEQ ID NO.: 911) of different length induce monocyte stimulation but have different levels of activity e.g., SEQ ID NO.: 433> SEQ ID NO.: 911. Poly A (SEQ ID NO.: 1095) as well as Poly C (SEQ ID NO.: 1096 (2178) sequences, in contrast, did not 5 lead to activation of monocytes (measured by the upregulation of CD80 at a concentration of 6 $\mu$ g/ml ODN).

*Example 8: Induction of cytokine release by non-CpG ODNs*

Next the ability of different T-rich ODNs to influence the cytokine milieu was examined. PBMC ( $3 \times 10^6$  cells/ml) were cultured for 24h with or without 6 $\mu$ g/ml of the 10 indicated ODNs or 1 $\mu$ g/ml LPS as positive control (n=2). After incubation supernatants were collected and TNF $\alpha$  measured by ELISA as described above and the results are shown in Fig. 11. PBMC were cultured with the indicated ODNs (1.0 $\mu$ g/ml) as described in Fig. 11 and IL-6 was measured in the supernatants by ELISA and the results are shown in Fig. 12.

Fig. 11 and 12 demonstrate that T-rich non-CpG and T-rich/CpG ODNs can 15 induce the secretion of the pro-inflammatory cytokines TNF $\alpha$  and IL-6. For both cytokines, ODN 5126 (SEQ ID NO.: 1058) was found in most assays to be as potent as ODN 2194 (SEQ ID NO.: 911). It is known that CpG ODNs influence the Th1/Th2 balance by preferentially inducing Th1 cytokines (Krieg, A. M. 1999. Mechanism and 20 applications of immune stimulatory CpG oligodeoxynucleotides. *Biochimica et Biophysica Acta* 933(1):1). To test whether T-rich ODN caused a similar shift to Th1 cytokines, IFN $\gamma$  production in PBMC was measured. In a first set of experiments, it was demonstrated that, as described for IL-6 and TNF $\alpha$ , ODNs SEQ ID NO.: 1058 and SEQ ID NO.: 911 induced the release of comparable amounts of this Th1 cytokine IFN $\gamma$ . In 25 addition, it was demonstrated that another pro-inflammatory cytokine, IL-1 $\beta$ , was released upon culture of PBMC with these two ODNs. Although the amount of these cytokines induced by the T-rich ODN lacking CpG motifs was less than when CpG ODN SEQ ID NO.: 246 was used the amounts induced by T-rich ODN were significantly higher than the control.

(2-1)

*Introduction:*

An optimal CpG motif for immune system activation in non-rodent vertebrates is described herein. A phosphodiester oligonucleotide containing this motif was found to strongly stimulate CD86, CD40, CD54 and MHC II expression, IL-6 synthesis and 5 proliferation of primary human B-cells. These effects required internalisation of the oligonucleotide and endosomal maturation. This CpG motif was associated with the sustained induction of the NF $\kappa$ B p50/p65 heterodimer and of the transcription factor complex activating protein-1 (AP-1). Transcription factor activation by CpG DNA was preceded by increased phosphorylation of the stress kinases c-jun NH<sub>2</sub> terminal kinase 10 (JNK) and p38, and of activating transcription factor-2 (ATF-2). In contrast to CpG, signaling through the B-cell receptor led to activation of extracellular receptor kinase (ERK) and to phosphorylation of a different isoform of JNK.

*Materials and Methods:*

Oligodeoxynucleotides: Unmodified (phosphodiester, PE) and modified 15 nuclease-resistant (phosphorothioate, PS) ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). The sequences used are provided in Table H. E. coli DNA and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, MO. Genomic DNA samples were purified by extraction with phenol-chloroform-isoamyl alcohol (25/24/1) and ethanol 20 precipitation. DNA was purified from endotoxin by repeated extraction with triton x-114 (Sigma Chemical Co., St. Louis, MO) and tested for endotoxin using the LAL-assay (LAL-assay BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml) and the high sensitivity assay for endotoxin described earlier (lower detection limit 0.0014 EU/ml) (Hartmann G., and Krieg A. M. 1999. CpG DNA and LPS induce distinct 25 patterns of activation in human monocytes. *Gene Therapy* 6:893). Endotoxin content of DNA samples was below 0.0014 U/ml. E. coli and calf thymus DNA were made single stranded before use by boiling for 10 minutes, followed by cooling on ice for 5 minutes. DNA samples were diluted in TE-buffer using pyrogen-free reagents.

30

Table H: Oligonucleotide panel used<sup>1</sup>

| Name (SEQ | Sequence 5' to 3' |
|-----------|-------------------|
|-----------|-------------------|

| ID NO)                     |               |  |
|----------------------------|---------------|--|
| Starting sequence          | PE 2079 (320) | <u>TCG ACG</u> TTC CCC CCC CCC CC                    |
| Middle base                | PE 2100 (341) | TCG GCG TTC CCC CCC CCC CC                           |
|                            | PE 2082 (323) | TCG CCG TTC CCC CCC CCC CC                           |
| Human CpG motif            | PE 2080 (321) | TCG TCG TTC CCC CCC CCC CC                           |
| S' flanking base           | PE 2105 (346) | GCG TCG TTC CCC CCC CCC CC                           |
|                            | PE 2107 (348) | ACG TCG TTC CCC CCC CCC CC                           |
|                            | PE 2104 (345) | CCG TCG TTC CCC CCC CCC CC                           |
| 3' flanking base           | PE 2098 (339) | TCG TCG CTC CCC CCC CCC CC                           |
|                            | PE 2099 (340) | TCG TCG GTC CCC CCC CCC CC                           |
|                            | PE 2083 (324) | TCG TCG ATC CCC CCC CCC CC                           |
| First CpG deleted          | PE 2108 (349) | CTG <u>TCG</u> TTC CCC CCC CCC CC                    |
| Second CpG deleted         | PE 2106 (347) | <u>TCG</u> TCA TTC CCC CCC CCC CC                    |
| Methylation                | PE 2095 (336) | TZG TZG TTC CCC CCC CCC CC                           |
|                            | PE 2094 (335) | TCG TCG TTC CCC CCC ZCC CC                           |
| Non-CpG control of<br>2080 | PE 2078 (319) | TGC TGC TTC CCC CCC CCC CC                           |
|                            | PE 2101 (342) | GGC CTT TTC CCC CCC CCC CC                           |
| PS form of 2080            | PS 2116 (357) | <u>TCG TCG</u> TTC CCC CCC CCC CC                    |
| Additional CpG motifs      | PE 2059 (300) | <u>TCG TCG</u> TTT TGT <u>CGT</u> TTT GTC <u>GTT</u> |
| Best PS                    | PS 2006 (246) | <u>TCG TCG</u> TTT TGT <u>CGT</u> TTT GTC <u>GTT</u> |
| Methylated 2006            | PS 2117 (358) | TZG TZG TTT TGT ZGT TTT GTZ GTT                      |

<sup>1</sup>PE, phosphodiester; PS, phosphorothioate; bold, base exchange; bold Z, methylated cytidine; underlined, CpG dinucleotides.

- Cell preparation and cell culture: Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) as described (Hartmann G., Krug A., Eigler A., Moeller J., Murphy J., Albrecht R., and 5 Endres S. 1996. Specific suppression of human tumor necrosis factor-alpha synthesis by antisense oligodeoxynucleotides. *Antisense Nucleic Acid Drug Dev* 6:291)). Cells were suspended in RPMI 1640 culture medium supplemented with 10 % (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, NY) (complete medium).
- 10 All compounds were purchased endotoxin-tested. Viability was determined before and after incubation with ODN by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometric analysis). In all experiments, 96 % to 99 % of PBMC were viable. Cells (final concentration  $1 \times 10^6$  cells/ml) were cultured in complete medium in a 5 % CO<sub>2</sub> humidified incubator at 37°C. Different oligonucleotides 15 (see table I, concentration as indicated in the figure legends), LPS (from salmonella typhimurium, Sigma Chemical Co., St. Louis, MO) or anti-IgM were used as stimuli. Chloroquine (5 µg/ml; Sigma Chemical Co., St. Louis, MO) was used to block endosomal maturation/acidification. At the indicated time points, cells were harvested for flow cytometry as described below.
- 20 For signal transduction studies, human primary B-cells were isolated by immunomagnetic cell sorting using the VARIOMACS technique (Miltenyi Biotec Inc., Auburn, CA) as described by the manufacturer. In brief, PBMC obtained from buffy coats of healthy blood donors (Elmer L. DeGowin Blood Center, University of Iowa) were incubated with a microbeads-conjugated antibody to CD19 and passed over a 25 positive selection column. Purity of B-cells was higher than 95%. After stimulation, whole cellular extracts (Western blot) and nuclear extracts (EMSA) for signal transduction studies were prepared.
- For CpG binding protein studies, Ramos cells (human Burkitt lymphoma B cell line, ATCC CRL-1923 or CRL-1596; Intervirology 5: 319-334, 1975) were grown in 30 complete medium. Untreated cells were harvested and cytosolic protein extracts were prepared and analyzed for the presence of CpG oligonucleotide binding proteins by EMSA and UV-crosslink as described below.

- Flow cytometry: Staining of surface antigens was performed as previously described (Hartmann G., Krug A., Bidlingmaier M., Hacker U., Eigler A., Albrecht R., Strasburger C. J., and Endres S. 1998. Spontaneous and cationic lipid-mediated uptake of antisense oligonucleotides in human monocytes and lymphocytes. *J Pharmacol Exp Ther* 285:920). Monoclonal antibodies to HLA-DR were purchased from Immunotech, Marseille, France. All other antibodies were purchased from Pharmingen, San Diego, CA: mABs to CD19 (B43), CD40 (5C3), CD54 (HA58), CD86 (233I (FUN-1)). IgG<sub>1,κ</sub> (MOPC-21) and IgG<sub>2b,κ</sub> were used to control for specific staining. Intracellular cytokine staining for IL-6 was performed as described (Hartmann G., and Krieg A. M. 1999. CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Therapy* 6:893). In brief, PBMC (final concentration  $1 \times 10^6$  cells/ml) were incubated in the presence of brefeldin A (final concentration 1 µg/ml, Sigma Chemical Co., St. Louis, MO). After incubation, cells were harvested and stained using a FITC-labeled mAB to CD19 (B43), a PE-labeled rat anti-human IL-6 mAb (MQ2-6A3, Pharmingen) and the Fix and Perm Kit (Caltag Laboratories, Burlingame, CA). Flow cytometric data of 5000 cells per sample were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Non-viable cells were excluded from analysis by propidium iodide staining (2 µg/ml). Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stanford, CA).
- Proliferation assay: CFSE (5-(and-6-) carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, USA) is a fluorescein-derived intracellular fluorescent label which is divided equally between daughter cells upon cell division. Staining of cells with CFSE allows both quantification and immunophenotyping (phycoerythrin-labeled antibodies) of proliferating cells in a mixed cell suspension.
- Briefly, PBMC were washed twice in PBS, resuspended in PBS containing CFSE at a final concentration of 5 µM, and incubated at 37°C for 10 minutes. Cells were washed three times with PBS and incubated for five days as indicated in the figure legends. Proliferating CD19-positive B-cells were identified by decreased CFSE content using flow cytometry.
- Preparation of whole cell, nuclear and cytosolic protein extracts: For Western blot analysis, whole cell extracts were prepared. Primary B-cells were treated with medium, the phosphodiester oligonucleotides 2080 (SEQ ID NO.: 321) or 2078 (SEQ ID

NO.: 319) at 30 µg/ml, or anti-IgM (10 µg/ml). Cells were harvested, washed twice with ice-cold PBS containing 1 mM Na<sub>3</sub>VO<sub>4</sub>, resuspended in lysis buffer (150 mM NaCl, 10 mM TRIS pH 7.4, 1 % NP40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml antipain, 5 mg/ml pepstatin, 50 µg/ml phenylmethylsulfonylfluoride (PMSF)), incubated for 15 min on ice and spun at 14000 rpm for 10 min. The supernatant was frozen at -80 C. For the preparation of nuclear extracts, primary B-cells were resuspended in hypotonic buffer (10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 0.05 % NP40, 1.5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml antipain, 5 mg/ml pepstatin). After 15 minutes incubation on ice, the suspension was centrifuged at 1000 x g for 5 minutes. The pelleted nuclei were resuspended in extraction buffer (20 mM HEPES (pH 7.9), 450 mM NaCl, 50 mM NaF, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 30 mg/ml leupeptin, 50 mg/ml aprotinin, 5 mg/ml antipain, 5 mg/ml pepstatin) and incubated on ice for one hour. The nuclear suspension was centrifuged for 10 minutes at 16,000 g at 4°C. Supernatant was collected and stored at -80°C. Cytosolic extracts for the CpG binding protein studies were prepared from unstimulated Ramos cells, which were lysed with hypotonic buffer as described for the preparation of the nuclear extract. After centrifugation, the supernatant was removed as cytoplasmic fraction and stored at -80°C. Protein concentrations were measured using a Bradford protein assay (Bio-Rad, Hercules, CA) according to the manufacturer.

Western blot analysis: Equal concentrations of whole cell protein extracts (25 µg/lane) were boiled in SDS sample buffer (50 mM Tris-Cl, pH 6.8; 1% β-mercaptoethanol; 2% SDS; 0.1% bromphenolblue; 10% glycerol) for 4 min before being subjected to electrophoresis on a 10 % polyacrylamide gel containing 0.1 % SDS (SDS-PAGE). After electrophoresis, proteins were transferred to Immobilon-P transfer membranes (Millipore Corp. Bedford, MA). Blots were blocked with 5 % nonfat dry milk. Specific antibodies against the phosphorylated form of extracellular receptor kinase (ERK), c-jun NH<sub>2</sub>-terminal kinase (JNK), p38 and activating transcription factor-2 (ATF-2) were used (New England BioLabs, Beverly, MA). Blots were developed in enhanced chemiluminescence reagent (ECL; Amersham International, Aylesbury, U.K.) according to the manufacturer's recommended procedure.

- Electrophoretic mobility shift assay (EMSA):** To detect the DNA-binding activity of the transcription factor activator protein-1 (AP-1) and NF $\kappa$ B, nuclear extracts (1  $\mu$ g/lane) were analyzed by EMSA using the dsODNs 5' GAT CTA GTG ATG AGT CAG CCG GAT C 3' (SEQ ID NO.: 838) containing the AP-1 binding sequence, and the 5 NF $\kappa$ B URE from the c-myc promotor region 5' TGC AGG AAG TCC GGG TTT TCC CCA ACC CCC C 3' (SEQ ID NO.: 1142), as probes. ODNs were end labeled with T4-polynucleotide kinase (New England Biolabs) and ( $\gamma$ - $^{32}$ P) ATP (Amersham, Arlington Heights, IL). Binding reactions were performed with 1  $\mu$ g nuclear protein extract in DNA-binding buffer (10 mM Tris-HCl (pH 7.5), 40 mM MgCl<sub>2</sub>, 20 mM EDTA, 1 mM dithiothreitol, 8% glycerol and 100 - 400 ng of poly (dI-dC) with 20.000 - 40.000 cpm labeled ODN in 10  $\mu$ l total volume. Specificity of the NF $\kappa$ B bands was confirmed by competition studies with cold oligonucleotides from unrelated transcription factor binding sites (10 - 100 ng). For the supershift assay, 2  $\mu$ g of specific antibodies for c-Rel, p50 and p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added into the 10 reaction mixture for 30 min before the radiolabeled probe was added. Following 15 incubation for 30 minutes at room temperature loading buffer was added and the probes were electrophoresed on a 6 % polyacrylamide gel in Tris-borate-EDTA running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0). Gels were dried and then autoradiographed.
- 20 **UV-crosslinking and denaturing protein electrophoresis:** Nuclear extracts were incubated with labeled phosphodiester oligonucleotide as described for the EMSA. DNA-protein complexes were crosslinked with UV-light in a Stratalinker (Stratagene) for 10 minutes. Probes were mixed with SDS-sample buffer, boiled for 10 minutes and loaded on a 7.5% SDS-PAGE. The gel was dried on Whatman paper and 25 autoradiographed. Plotting the distance against the molecular weight of the marker proteins yielded a standard curve which was used to calculate the approximate molecular weight of the crosslinked protein-ODN complexes. The molecular weight of the oligonucleotide was subtracted from this value to give the size.

30 *Example 9: Identification of an Optimal CpG motif for use alone or in combination with a T-rich ODN*

Phosphorothioate oligonucleotides containing the murine CpG motif GACGTT (SEQ ID NO.: 1143) (for example 1826 (SEQ ID NO.: 69)) and used at concentrations which are active in murine B-cells (Yi A. K., Chang M., Peckham D. W., Krieg A. M., and Ashman R. F. 1998. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J Immunol* 160:5898), have showed little or no immunostimulatory activity on human immune cells. At higher concentrations this ODN was found to demonstrate some stimulatory effect on human B cells.

In earlier studies on B-cell activation in mice, it was found that a CpG-dinucleotide flanked by two 5' purines and two 3' pyrimidines and preferably the 6mer motif 5' GACGTT 3' (SEQ ID NO:1143) was optimal for a phosphodiester oligonucleotide to be active (Krieg A. M., et al. 1995 *Nature* 374:546, Yi A. K., Chang M., et al.. 1998 *J Immunol* 160:5898).

In order to identify an optimal motif for stimulation of an immune response in humans and non-rodent vertebrates we designed a series of ODN and tested their activity. First we designed a 20 mer phosphodiester oligonucleotide with a TC dinucleotide at the 5' end preceding the optimal murine CpG motif 5' GACGTT 3' (SEQ ID NO.: 1143) and followed by a poly C tail (2079: 5' TCG ACG TTC CCC CCC CCC CC 3'(SEQ ID NO.: 320)). This oligonucleotide if added to human primary B-cells under the same conditions as found to be optimal for E. coli DNA (repeated addition at 0 hours, 4 hours and 18 hours; 30 µg/ml for each time point) stimulated high levels of CD86 expression on human primary B-cells after two days. To determine the structure-function relationship of the CpG motifs, we replaced the bases adjacent to the CpG dinucleotides while maintaining the two CpG dinucleotides within the sequence. Exchange of the adenine located between both CpG dinucleotides by thymidine (2080 (SEQ ID NO.: 321)) resulted in slightly higher activity. Replacement by guanosine (2100 (SEQ ID NO.: 341)) or cytidine (2082 (SEQ ID NO.: 323)) at this position showed no major changes compared to 2079 (SEQ ID NO.: 320). In contrast, replacement of the thymidine 3' to the second CpG dinucleotide by the purines guanosine (2099 (SEQ ID NO.: 340)) or adenine (2083 (SEQ ID NO.: 324)) resulted in a major drop in activity of the oligonucleotide, while the pyrimidine cytidine caused only a minor decrease. The thymidine immediately 5' to the first CpG dinucleotide was also important.

Replacement of the thymidine by any other base (2105 (SEQ ID NO.: 346), guanosine; 2107 (SEQ ID NO.: 348), adenine; 2104 (SEQ ID NO.: 345), cytidine) led to a marked decrease in activity of the oligonucleotide. Elimination of the first (2108 (SEQ ID NO.: 349)) or the second (2106 (SEQ ID NO.: 347)) CpG dinucleotide also partially reduced 5 the activity.

The addition of more 5' GTCGTT 3' (SEQ ID NO.: 1144) CpG motifs to the phosphodiester oligonucleotide containing the 8mer duplex CpG motif (5' TCGTCGTT 3' (SEQ ID NO:1145) , 2080 (SEQ ID NO.: 321)) did not further enhance CD86 expression on B-cells (2059 (SEQ ID NO.: 300)). An oligonucleotide with the same 10 sequence as 2080 (SEQ ID NO.: 321) but with a phosphorothioate backbone showed no activity above background (2116 (SEQ ID NO.: 357)). This was surprising since the phosphorothioate backbone has been reported to greatly stabilize oligonucleotides and enhance CpG-induced stimulation (Krieg A. M., Yi A. K., Matson S., Waldschmidt T. J., Bishop G. A., Teasdale R., Koretzky G. A., and Klinman D. M. 1995. CpG motifs in 15 bacterial DNA trigger direct B-cell activation. *Nature* 374:546). We therefore performed further structure-function analysis of phosphorothioate oligonucleotides containing the 5' GTCGTT 3' (SEQ ID NO: 1144) and 5' TCGTCGTT 3' (SEQ ID NO:1145) motifs, which showed that additional CpG motifs (2006 (SEQ ID NO.: 246)) tended to increase the activity of phosphorothioate oligonucleotides.

20 Purified B-cells isolated from peripheral blood by immunomagnetic cell sorting were activated by CpG DNA to the same extent as unpurified B-cells within PBMC. Thus, activation of B-cells is a primary response and not a secondary effect caused by cytokines secreted by other cells.

In addition to the co-stimulatory molecule CD86, the functional stage of B-cells 25 is characterized by other surface markers. For example, activated T helper cells stimulate B-cells by CD40 ligation, the intercellular adhesion molecule-1 (ICAM-1, CD54) mediates binding to other immune cells, and major histocompatibility complex II (MHC II) is responsible for antigen presentation. We found that B cell expression of CD40, CD54 and MHC II was upregulated by the CpG oligonucleotide 2080 (SEQ ID NO.: 321). The non-CpG control oligonucleotide 2078 (SEQ ID NO.: 319) showed no activity 30 compared to medium alone.

When PBMC were incubated for 5 days in the presence of 2080 (SEQ ID NO.: 321) (added at 0 hours, 4 hours, 18 hours and every subsequent morning), it was intriguing that a subpopulation of lymphocytes increased in cell size (FSC) and became more granular (SSC). To examine if this subpopulation represented proliferating B-cells, 5 we stained freshly isolated PBMC with CFSE (5-(and-6-) carboxyfluorescein diacetate succinimidyl ester) at day 0 and incubated them for 5 days with 2080 (SEQ ID NO.: 321) as above. CFSE is a fluorescent molecule that binds irreversibly to cell proteins. Each cell division decreases CFSE stain by 50 %. Cells staining low with CFSE (proliferating cells) were found to be mainly CD19-positive B-cells. The oligonucleotide 2080 (SEQ 10 ID NO.: 321) induced 60 to 70 % of CD19 positive B-cells to proliferate within 5 days. The control oligonucleotide 2078 (SEQ ID NO.: 319) induced less than 5 % of B-cells to proliferate. Proliferating B-cells (CFSE low) showed a larger cell size (FSC) and higher granularity.

Proliferating B-cells expressed higher levels of CD86 than non-proliferating cells 15 (not shown). In agreement with this finding, the oligonucleotide panel tested above for induction of CD86 expression resulted in an almost identical pattern of B-cell proliferation. Replacement of the 3' thymidine reduced activity more than changing the thymidine in the middle position.

20           *Example 10: B-cell activation requires endosomal maturation/acidification*

It has previously been shown that chloroquine, an inhibitor of endosomal acidification, blocks CpG-mediated stimulation of murine antigen presenting cells and B-cells, while not influencing LPS-mediated effects (Hacker H., et al 1998 *Embo J* 17:6230, Yi A. K. et al 1998 *J Immunol* 160:4755, Macfarlane D. E., and Manzel L. 25 1998 *J Immunol* 160:1122). We found that the addition of 5 µg/ml chloroquine, completely blocked CpG DNA-mediated induction of CD86 expression on primary B-cells (MFI CD86: 2006 (SEQ ID NO.: 246), 4.7 vs 1.4; E. coli DNA, 3.4 vs. 1.4; medium only, 0.9; n=4). Furthermore, chloroquine completely inhibited the induction of B-cell proliferation by the phosphorothioate oligonucleotide 2006 (SEQ ID NO.: 246) 30 measured with the CFSE proliferation assay as well as with the standard. These results suggest that as with murine cells, activation of human B-cells by CpG DNA requires the uptake of DNA in endosomes and subsequent endosomal acidification.

*Example II: Analysis of sub-cellular events resulting upon human B cell stimulation with optimal human ODN.*

Since the CpG motif requirement for maximal B-cell activation is substantially different between mouse (GACGTT) (SEQ ID NO:1143) and humans (TCGTCGTT) (SEQ ID NO:1145), we were interested if the basic intracellular signaling events are comparable. Rapid induction of NF $\kappa$ B binding activity has been found earlier in murine B-cells and macrophages (Stacey K. J., et al 1996 *J Immunol* 157:2116, Yi A. K et al 1998 *J Immunol* 160:4755). To investigate the NF $\kappa$ B response to CpG DNA in humans, 10 human primary B-cells were isolated from peripheral blood by immunomagnetic cell sorting and incubated with the CpG oligonucleotide 2080 (SEQ ID NO.: 321), the non-CpG control oligonucleotide 2078 (SEQ ID NO.: 319), or medium. At the indicated time points, cells were harvested and nuclear extracts were prepared. In the presence of CpG oligonucleotide, NF $\kappa$ B binding activity was increased within one hour and maintained up 15 to 18 hours (latest time point examined). The non-CpG control oligonucleotide 2078 (SEQ ID NO.: 319) did not show enhanced NF $\kappa$ B activity compared to cells incubated with medium only. The NF $\kappa$ B band was identified by cold competition, and shown to consist of p50 and p65 subunits by supershift assay.

The activating protein-1 (AP-1) transcription factor is involved in the regulation 20 of immediate early genes and cytokine expression (Karin M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 270:16483). In murine B-cells, AP-1 binding activity is induced in response to CpG DNA (Yi A. K., and Krieg A. M. 1998. Rapid induction of mitogen-activated protein kinases by immune stimulatory 25 CpG DNA. *J Immunol* 161:4493). To determine whether this transcription factor would also be induced by CpG DNA in humans, we examined AP-1 DNA binding activity in human primary B-cells. Cells were incubated with the CpG oligonucleotide 2080 (SEQ ID NO.: 321) or the control oligonucleotide 2078 (SEQ ID NO.: 319). Nuclear extracts were prepared and the AP-1 binding activity was analyzed by EMSA. AP-1 binding 30 activity was enhanced within one hour, and increased up to 18 hours (latest time point examined), showing a sustained response.

Since AP-1 activity is induced by many stimuli (Angel P., and Karin M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.

*Biochim Biophys Acta* 1072:129), we were interested in signal transduction pathways upstream of AP-1. The AP-1 transcription factor complex integrates different mitogen activated protein kinase (MAPK) pathways (Karin M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 270:16483). Western blots 5 were performed using whole cell extracts from primary B-cells incubated with the CpG oligonucleotide 2080 (SEQ ID NO.: 321), the control 2078 (SEQ ID NO.: 319), or medium only. Specific antibodies to the phosphorylated form of JNK, p38, ATF-2 and ERK were used. Strong induction of JNK phosphorylation was found 30 min and 60 min after exposure to CpG-DNA, while the non-CpG oligonucleotide showed no activity 10 above background. The protein kinase p38, another stress activated protein kinase (SAPK), was also phosphorylated in response to CpG DNA within 60 min. ATF-2, a substrate of both p38 and JNK (Gupta S., Campbell D., Derijard B., and Davis R. J. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267:389) and a component of the AP-1 complex, showed weak phosphorylation 15 after 30 min which increased after 60 min. CpG DNA failed to induce substantial phosphorylation of ERK. In contrast, anti-IgM, stimulating the B-cell receptor, did trigger phosphorylation of ERK. Anti-IgM activated different isoforms of JNK than CpG DNA.

*Example 12: Assay for in vivo adjuvant activity.*

20 An *in vitro* screening assay to identify ODN useful as an adjuvant *in vivo* in humans and other non-rodent animals was developed. Since we saw not only quantitative but also qualitative differences in activities of different CpG ODN in mice, we first screened a panel of CpG and non-CpG control ODN on mouse cells to find *in vitro* assays with reliable and strong correlation to *in vivo* adjuvant activity with hepatitis 25 B surface antigen (HBsAg). We then systematically tested a panel of more than 250 ODN in corresponding human assays to identify sequences with *in vitro* immunostimulatory activity. We next examined if the ODN with the highest activity in these human assays also activate B cell proliferation in chimpanzees and monkeys, and finally, if they are active as adjuvants with HBsAg in chimpanzees and cynomolgus 30 monkeys *in vivo*. These studies revealed that the sequence, number and spacing of individual CpG motifs contribute to the immunostimulatory activity of a CpG phosphorothioate ODN. An ODN with a TC dinucleotide at the 5' end followed by three

6mer CpG motifs (5' GTCGTT 3') separated by TT dinucleotides consistently showed the highest activity for human, chimpanzee, and rhesus monkey leukocytes. Chimpanzees or monkeys vaccinated once against hepatitis B with this CpG ODN adjuvant developed 15 times higher anti-HBs antibody titers than those receiving vaccine alone.

*Materials and Methods*

**Oligodeoxynucleotides:** Phosphorothioate-modified ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). ODN were tested for endotoxin using the LAL-assay (LAL-assay BioWhittaker, Walkersville, MD; lower detection limit 0.1 EU/ml). For *in vitro* assays, ODN were diluted in TE-buffer (10 mM Tris, pH 7.0, 1 mM EDTA), and stored at -20° C. For *in vivo* use, ODN were diluted in phosphate buffered saline (0.1 M PBS, pH 7.3) and stored at 4°C. All dilutions were carried out using pyrogen-free reagents.

**Mouse spleen cell cultures:** Spleens were removed from 6-12 week old female BALB/c (The Jackson Laboratory),  $2 \times 10^6$  splenocytes were cultured with 0.2  $\mu$ M ODN for 4 hours (TNF- $\alpha$ ) or 24 hours (IL-6, IFN- $\gamma$ , IL-12), and cytokines were detected by ELISA as previously described (Yi A. K., Klinman D. M., Martin T. L., Matson S., and Krieg A. M. 1996. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol* 157:5394). To evaluate CpG-induced B cell proliferation, spleen cells were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lympholyte M® (Cedarlane Laboratories, Hornby, ON, Canada), cultured for 44 hours with the indicated ODN, and then pulsed for 4 hours with 1  $\mu$ Ci of  $^3$ H thymidine as described previously (Krieg A. M., Yi A. K., Matson S., Waldschmidt T. J., Bishop G. A., Teasdale R., Koretzky G. A., and Klinman D. M. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546). To examine NK cell lytic activity murine spleen cells were depleted of B cells using magnetic beads coated with goat anti-mouse Ig as previously detailed (Ballas Z. K., and Rasmussen W. 1993. Lymphokine-activated killer cells. VII. IL-4 induces an NK1.1 $^+$ CD8  $\alpha^+ \beta^-$  TCR- $\alpha\beta$  B220 $^+$  lymphokine-activated killer subset. *J Immunol* 150:17). Cells were cultured at  $5 \times 10^6$ /well in 24-well plates and harvested at 18 hours for use as effector cells in a standard 4 hour  $^{51}$ Cr-release assay

against YAC-1 target cells. One unit (LU) was defined as the number of cells needed to effect 30 % specific lysis.

- Immunization of mice against HBsAg and evaluation of the humoral response: Groups of 6-8 week old female BALB/c mice (n = 5 or 10, Charles River, 5 Montreal, QC) were immunized against HBsAg as previously described (Davis H. L., et al 1998 *J Immunol* 160:870). In brief, each mouse received a single IM injection of 50 µl PBS containing 1 µg recombinant HBsAg (Medix Biotech, Foster City, CA) and 10 µg of CpG ODN or non-CpG ODN as a sole adjuvant or combined with alum (Alhydrogel "85", Superfos Biosector, Vedbaek, Denmark; 25 mg Al<sup>3+</sup>/mg HBsAg).
- 10 Control mice were immunized with HBsAg without adjuvant or with alum. Plasma was recovered from mice at various times after immunization and Abs specific to HBsAg (anti-HBs) were quantified by end-point dilution ELISA assay (in triplicate) as described previously (Davis H. L et al 1998 *J Immunol* 160:870). End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD450) two times 15 higher than that of non-immune plasma with a cut-off value of 0.05.

- Isolation of primate PBMC and cell culture: Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers, chimpanzees or rhesus or cynomolgus monkeys by Ficoll-hypaque density gradient centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) as described (Hartmann G., et al 20 1996 *Antisense Nucleic Acid Drug Dev* 6:291). Cells were suspended in RPMI 1640 culture medium supplemented with 10 % (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, NY) (complete medium). Cells (final concentration 1 × 10<sup>6</sup> cells/ml) were cultured in complete medium in a 5 % CO<sub>2</sub> humidified incubator at 37°C. ODN and LPS (from *Salmonella typhimurium*, Sigma 25 Chemical Co., St. Louis, MO) or anti-IgM were used as stimuli. For measurement of human NK lytic activity, PBMC were incubated at 5 × 10<sup>6</sup>/well in 24-well plates. Cultures were harvested after 24 hours, and cells were used as effectors in a standard 4 hours <sup>51</sup>Cr-release assay against K562 target cells as previously described (Ballas Z. K., 30 Rasmussen W. L., and Krieg A. M. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* 157:1840; Ballas Z. K., and Rasmussen W. 1993. Lymphokine-activated killer cells. VII. IL-4

induces an NK1.1<sup>+</sup>CD8  $\alpha^+ \beta^-$  TCR- $\alpha\beta$  B220<sup>+</sup> lymphokine-activated killer subset (*J Immunol* 150:17). For B cell proliferation, 1  $\mu$ Ci of  $^3$ H thymidine was added 18 hours before harvest, and the amount of  $^3$ H thymidine incorporation was determined by scintillation counting at day 5. Standard deviations of the triplicate wells were < 5%.

5 Flow cytometry on primate PBMC: Surface antigens on primate PBMC were stained as previously described (Hartmann G et al 1998 *J Pharmacol Exp Ther* 285:920). Monoclonal antibodies to CD3 (UCHT1), CD14 (M5E2), CD19 (B43), CD56 (B159), CD69 (FN50) and CD86 (2331 (FUN-1)) were purchased from Pharmingen, San Diego, CA. IgG<sub>1,k</sub> (MOPC-21) and IgG<sub>2b,k</sub> (Hartmann G et al 1999 *PNAS* 96:9305)

10 were used to control for non-specific staining. NK cells were identified by CD56 expression on CD3, CD14 and CD19 negative cells, whereas B cells were identified by expression of CD19. Flow cytometric data from 10000 cells per sample were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA). The viability of cells within the FSC/SSC gate used for analysis was examined by propidium iodide staining (2  $\mu$ g/ml) and found to be higher than 98 %. Data were analyzed using

15 the computer program FlowJo (version 2.5.1, Tree Star, Inc., Stanford, CA).

Immunization of chimpanzees and cynomolgus monkeys against HBsAg and evaluation of the humoral response: Fourteen cynomolgus monkeys (2.0–3.5 kg) were immunized with a pediatric dose of Engerix-B (SmithKline Beecham Biologicals, Rixensart, BE) containing 10  $\mu$ g HBsAg adsorbed to alum (25 mg Al<sup>3+</sup>/mg HBsAg). This was administered alone (n=5), or combined with CpG ODN 1968 (n=5, 500  $\mu$ g) or CpG ODN 2006 (SEQ ID NO.: 246) (n=4, 150  $\mu$ g). Four chimpanzees (10-20 kg) were immunized in the same fashion with two receiving control vaccine (Engerix-B only) and two receiving experimental vaccine (Engerix-B plus 1 mg CpG ODN 2006). All vaccines were administered IM in the right anterior thigh in a total volume of 1 ml. Monkeys were maintained in the animal facility of the Primate Research Center (Bogor, Indonesia) and chimpanzees were housed at Bioqual (Rockville, MD). Animals were monitored daily by animal care specialists. No symptoms of general ill health or local adverse reactions at the injection site were noted. Plasma was recovered by IV puncture prior to and at various times after immunization and was stored frozen (-20°C) until assayed for antibodies. Anti-HBs antibodies were detected using a commercial ELISA kit (Monolisa Anti-HBs; Sanofi-Pasteur, Montreal, QC) and titers were expressed in mIU/ml based on

comparison with WHO defined standards (Monolisa Anti-HBs Standards; Sanofi-Pasteur).

*Results*

Identification of CpG ODN with different profiles of *in vitro* immune activities:

- 5 Our studies showed that the precise bases on the 5' and 3' sides of a CpG dinucleotide within a CpG motif may have an impact on the level of immune activation of a synthetic ODN, but it has been unclear whether different CpG motifs might display different immune effects. To evaluate this possibility, we tested a panel of CpG ODN for their ability to induce NK lytic activity, B cell proliferation, and to stimulate synthesis of
- 10 TNF- $\alpha$ , IL-6, IFN- $\gamma$  and IL-12 in murine spleen cells. Immunostimulatory activity of ODN without CpG motifs (ODN 1982 (SEQ ID NO.: 225), ODN 1983 (SEQ ID NO.: 226)) was negative or weak compared to CpG ODN. ODN with non optimal CpG motifs (ODN 1628 (SEQ ID NO.: 767), ODN 1758 (SEQ ID NO.: 1)) were less active than ODN containing CpG motifs flanked by two 5' purines and two 3' pyrimidines
- 15 (ODN 1760 (SEQ ID NO.: 3), ODN 1826 (SEQ ID NO.: 69), ODN 1841 (SEQ ID NO.: 84)). ODN 1826 containing two optimal murine CpG motifs (5' GACGTT 3') (SEQ ID NO.: 1143) had the highest activity for 5 of 6 measured endpoints. Except for ODN 1628, all ODN showed a generally similar pattern of activity (NK cell-mediated lysis, B cell proliferation, IL-12, IL-6, TNF  $\alpha$ , IFN- $\gamma$ ). Of note, ODN 1628, which was unique in this
- 20 panel for containing two G-rich regions, showed preferential induction of IFN- $\gamma$  synthesis but relatively low stimulation of the other activities.

Identification of *in vitro* assays which correlate with *in vivo* adjuvant activity:

- Since adjuvant activity is an *in vivo* endpoint, we were interested in identifying *in vitro* assays that would predict the adjuvant activity of a CpG ODN *in vivo*. The same ODN used for *in vitro* endpoints therefore were tested for their adjuvant activity to immunize mice against HBsAg. This was carried out both with ODN alone and with ODN combined with alum, since earlier studies had shown strong synergy for CpG ODN and alum adjuvants (PCT Published Patent Application WO98/40100).

- 30 BALB/c mice immunized with HBsAg without adjuvant attained only low titers of anti-HBs by 4 weeks, and this was not affected by addition of control ODN. In contrast, addition of CpG ODN raised anti-HBs titers by 5 to 40 fold, depending on the

sequence used. When alum was added, titers of anti HBs were approximately 6 times higher than with HBsAg alone. Specifically, control ODN had no effect and the various CpG ODN augmented these titers 2 to 36 fold. Results obtained with the different ODN alone correlated very strongly ( $r = 0.96$ ) with those obtained using the same ODN plus 5 alum. When linear regression was performed, a very high degree of correlation was found between certain *in vitro* assays and *in vivo* augmentation of anti-HBs titers. Of all the *in vitro* endpoints examined, the induction of NK lytic activity showed the best 10 correlation to *in vivo* adjuvant activity (without alum,  $r = 0.98$ ; with alum,  $r = 0.95$ ;  $p < 0.0001$ ). A good correlation regarding adjuvant activity was also obtained for B-cell 15 stimulation ( $r = 0.84$  and  $0.7$ ), as well as secretion of TNF- $\alpha$  ( $r = 0.9$  and  $0.88$ ), IL-12 ( $r = 0.88$  and  $0.86$ ) and IL-6 ( $r = 0.85$  and  $0.91$ ). The one *in vitro* assay that did not correlate well with the *in vivo* results was IFN- $\gamma$  secretion ( $r = 0.57$  and  $0.68$ ). These data demonstrate that *in vitro* assays for NK lytic activity, B cell activation and production of TNF- $\alpha$ , IL-6 and IL-12 provide valuable information *in vitro* to predict the adjuvant 15 activity of a given ODN *in vivo*.

Screening of a phosphorothioate ODN panel to activate human NK cells: In previous studies we found that synthesis of inflammatory cytokines by human PBMC is induced by extremely low amounts of endotoxin (induced TNF- $\alpha$  secretion is detectable with just 6 pg/ml endotoxin, 2 logs more sensitive than murine immune cells). In 20 contrast, activation of human B cells and induction of human NK cell lytic activity with endotoxin is low even at high endotoxin concentrations. Based on these results we selected activation of NK cells (lytic activity and CD69 expression) and B cells (proliferation and CD86 expression) as the most highly specific and reproducible assays with low inter-subject variability and used these assays for *in vitro* screening of a pool of 25 ODN.

First we studied the effect of phosphorothioate ODN containing various combinations and permutations of CpG motifs on NK cell-mediated lysis of target cells. For clarity and ease of presentation, only data with selected representative CpG and control ODN are shown. Human PBMC were incubated with different phosphorothioate 30 ODN (6  $\mu$ g/ml) for 24 hours and tested for their ability to lyse  $^{51}\text{Cr}$ -labeled K562 cells. ODN with two 6-mer CpG motifs (either 5' GACGTT 3' (SEQ ID NO.: 1143) or 5' GTCGTT 3' (SEQ ID NO.: 1144)) in combination with a TpC at the 5'end of the ODN

- (ODN 1840 5' TCCATGTCGTTCTGTCGTT 3' (SEQ ID NO.: 83), ODN 1851 5' TCCTGACGTTCTGACGTT 3' (SEQ ID NO.: 94) or with at least three 6-mer motifs without a TpC at the 5' end (ODN 2013 (SEQ ID NO.: 253)) show intermediate activity.
- High activity was found when the 5' TpC directly preceded a 6-mer human CpG motif
- 5    (5' TCGTCGTT 3' (SEQ ID NO.: 1145) (in SEQ ID NO.: 246)) and was followed by two 6-mer motifs (ODN 2005 (SEQ ID NO.: 245), ODN 2006 (SEQ ID NO.: 246) and ODN 2007 (SEQ ID NO.: 247)). The best results were obtained when the 6-mer CpG motifs were separated from each other and from the 5' 8-mer CpG motif by TpT (ODN 2006 (SEQ ID NO.: 246)).
- 10      Expression of the activation marker CD69 is rapidly upregulated on the surface of NK cells subsequent to stimulation. To confirm the results from the NK cell lysis assay, PBMC were incubated for 18 hours with ODN (2 µg/ml). CD69 expression was examined on CD56 positive NK cells (CD3, CD14 and CD19 negative). Although induction of CD69 expression was less sequence restricted than stimulation of NK cell
- 15      functional activity, control ODN (ODN 1982, ODN 2116, ODN 2117, ODN 2010) showed only low activity similar to background levels. ODN with two human CpG motifs separated by 5' TTTT 3' (ODN 1965 (SEQ ID NO.: 208)) or four human CpG motifs without spacing (ODN 2013 (SEQ ID NO.: 253)) were relatively more active at inducing CD69 expression than at stimulating NK cell lytic activity. Optimal NK cell
- 20      functional activity, as well as CD69 expression, was obtained with ODNs containing a TpC dinucleotide preceding the human CpG motif, and additional human motifs within the sequence (ODN 2006 (SEQ ID NO.: 246), ODN 2007 (SEQ ID NO.: 247)).

Activity of phosphorothioate ODN for stimulating human B cells: In preliminary experiments we found that the percentage of proliferating B cells (CFSE assay, see methods section) correlated with the surface expression of the co-stimulatory CD86 on B cells, as measured by flow cytometry. Thus we used CD86 expression on B cells to screen a panel of ODN for their immunostimulatory activity. PBMC were incubated with 0.6 µg/ml ODN. Expression of CD86 (mean fluorescence intensity, MFI) was examined on CD19 positive B cells. A poly C ODN (ODN 2017 (SEQ ID NO.: 257)) or ODN

25      without CpG dinucleotides (ODN 1982 (SEQ ID NO.: 225)) failed to stimulate human B cells under these experimental conditions. A phosphorothioate ODN (ODN 2116 (SEQ ID NO.: 256)) with one optimal human CpG motif preceded by a TpC (5' TCGTCGTT

3' (SEQ ID NO: 1145) (in SEQ ID NO.: 246)) was inactive. The presence of one human 6-mer CpG motif (5' GTCGTT 3' (SEQ ID NO.: 1144)) had no activating effect. Two of these CpG motifs within the sequence showed no (ODN 1960 (SEQ ID NO.: 203), ODN 2016 (SEQ ID NO.: 256)) or intermediate (ODN 1965 (SEQ ID NO.: 208))  
5 activity dependent on the sequence context. If the ODN was composed of three or four copies of this motif (ODN 2012 (SEQ ID NO.: 252), ODN 2013 (SEQ ID NO.: 253), ODN 2014 (SEQ ID NO.: 254)), intermediate activity on B cells could be detected. The combination of the human 8-mer CpG motif on the 5' end of the ODN with two 6-mer CpG motifs (ODN 2005 (SEQ ID NO.: 245), ODN 2006 (SEQ ID NO.: 246), ODN 2007  
10 (SEQ ID NO.: 247), ODN 2102 (SEQ ID NO.: 343), ODN 2103 (SEQ ID NO.: 344)) led to a considerable increase in the ability of the ODN to stimulate B cells. The spacing between the single motifs was critical. The separation of CpG motifs by TpT was preferable (ODN 2006 (SEQ ID NO.: 246)) compared to unseparated CpG motifs (ODN 2005 (SEQ ID NO.: )); also compare ODN 1965 (SEQ ID NO.: 208) to ODN 1960 (SEQ  
15 ID NO.: 203)). The human 6-mer CpG motif (5' GTCGTT 3') was better than the optimal mouse 6-mer CpG motif (5' GACGTT 3' (SEQ ID NO.: 246)) when combined with the human 8-mer CpG motif on the 5' end (ODN 2006 vs. ODN 2102 (SEQ ID NO.: 343) and ODN 2103 (SEQ ID NO.: 344)). A (TCG)<sub>poly</sub> ODN was inactive or only weakly active, as were ODN containing CpG dinucleotides flanked by guanines or other  
20 CpG dinucleotides (ODN 2010 (SEQ ID NO.: 250)). Taken together, the findings for NK cells and B cells showed consistently that of the ODN tested, ODN 2006 (SEQ ID NO.: 246) has the highest immunostimulatory activity on human immune cells.

Comparative analysis of potency of CpG phosphorothioate ODNs in different primates: Different CpG motifs are optimal to activate murine and human immune cells.  
25 Furthermore, the number and location of CpG motifs within an active phosphorothioate ODN is different in mice and humans. We were interested to know if CpG phosphorothioate ODN show a similar activity among different species of primates. We compared a panel of CpG ODN for their ability to induce B cell proliferation in humans, chimpanzees and rhesus or cynomolgus monkeys. The capability of ODN to stimulate  
30 human B cell proliferation (Table J) correlated well with their ability to induce CD86 expression on B cells. ODN 2006 (SEQ ID NO.: 246), which showed the highest activity in human B cells and NK cells, was also the most active in stimulating chimpanzee and

rhesus monkey B cell proliferation (Table J). ODN 1968 (SEQ ID NO.: 211) and ODN 2006 (SEQ ID NO.: 246) gave the highest activation of cynomolgus monkey B-cells *in vitro* (SI of 25 and 29 respectively at 6 µg ODN/ml). Surprisingly, CpG ODN 2007 (SEQ ID NO.: 247), which displayed similarly high activity as the optimal ODN 2006 5 (SEQ ID NO.: 246) in human cells, did not stimulate Rhesus monkey or chimpanzee B cell proliferation, and the ODN 1968 (SEQ ID NO.: 211) showed low activity. CpG ODN originally identified with high activity in mice (ODN 1760 (SEQ ID NO.: 3), ODN 1826 (SEQ ID NO.: 69)) showed little activity in monkeys (Table J).

Table J: Proliferative response of PBMC to phosphorothioate CpG ODN in primates

10

|                               | Humans   | Chimpanzee | Rhesus monkey |
|-------------------------------|----------|------------|---------------|
| No addition                   | 0.5+-0.1 | 0.5+-0.1   | 0.5+-0.0      |
| ODN 1760<br>(SEQ ID NO.: 3)   | 23+-7    | 0.3+-0.1   | 0.5+-0.3      |
| ODN 1826<br>(SEQ ID NO.: 69)  | 0.8+-0.1 | 0.4+-0.1   | 0.6+-0.1      |
| ODN 1968<br>(SEQ ID NO.: 211) | 35+-9    | 20.0+-3.8  | 1.9+-0.7      |
| ODN 1982<br>(SEQ ID NO.: 225) | 9.7+-1.1 | 2.5+-1.1   | 0.7+-0.1      |
| ODN 2006<br>(SEQ ID NO.: 246) | 58+-8    | 27.4+-8.9  | 6.3+-3.3      |
| ODN 2007<br>(SEQ ID NO.: 247) | 47+-11   | 0.5+-0.1   | 0.4+-0.2      |

PBMC were prepared from peripheral blood and incubated with ODN (0.6 µg/ml) as indicated for five days. Proliferation was measured by uptake of  $^3\text{H}$ /thymidine (cpm/1000) during the last 18 hours. More than 95 % of proliferating cells were B-cells as determined using the CFSE assay. Four human probands, six chimpanzees and two rhesus monkeys were tested.

In vivo adjuvant activity of CpG ODN in chimpanzees and cynomolgus monkeys: In order to evaluate whether CpG ODN with strong *in vitro* stimulatory effects on primate cells had detectable adjuvant activity *in vivo*, Cynomolgus monkeys and chimpanzees were immunized with Engerix B, which comprises HBsAg adsorbed to alum, alone or with added ODN 1968 (500 µg) or ODN 2006 (SEQ ID NO.: 246) (1 mg) respectively. Compared to controls not receiving CpG ODN, anti-HBs titers at 4 weeks post-prime and 2 weeks post-boost were 66- and 16-fold higher respectively in the monkeys, and 15- and 3-fold higher in the chimpanzees (Table K). Thus a clear adjuvant effect of CpG ODN was seen, and this was particularly striking after a single immunization.

Table K Anti-HBs responses in primates immunized against HBsAg with CpG ODN<sup>3</sup>

| <u>Primate species</u> | n | CpG ODN                               | 4 wks post-prime | 2 wks post-boost |
|------------------------|---|---------------------------------------|------------------|------------------|
| Cynomolgus monkey      | 5 | None                                  | 15 ± 44          | 4880 ± 13113     |
|                        | 5 | ODN 1968 (500 µg)<br>(SEQ ID NO. 211) | 995 ± 1309       | 76449 ± 42094    |
| Chimpanzee             | 2 | None                                  | 6, 11            | 3712, 4706       |
|                        | 2 | ODN 2006 (1 mg)<br>(SEQ ID NO. 246)   | 125, 135         | 9640, 16800      |

<sup>3</sup>Animals were immunized by IM injection of Engerix B containing 10 µg HBsAg adsorbed to alum, alone or with added CpG ODN. Cynomolgus monkeys were boosted at 10 wks and chimpanzees were boosted at 4 wks post-prime. Anti-HBs was determined by ELISA assay; values for monkeys are GMT ± SEM (n=5) whereas individual values for the two chimpanzees in each group are provided.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the

scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the  
5 invention.

I claim: